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**Study the Effect of Zearalenone and Kallistatin in Sex Hormone
Homeostasis among Patients with Polycystic Ovary Syndrome**

A thesis

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Kerbala, in Partial Fulfilment of the Requirements for the Degree of
Master in Clinical Chemistry and Biochemistry

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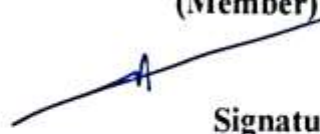


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Dedication

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To Allah — who heard my silent prayers, guided my steps through the darkest paths, and made this achievement possible by His mercy and will.

To the soul of my father — your absence left a silence only love can fill. You remain my strength and inspiration. I hope this work makes you proud.

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List of Abbreviations

Abbreviation	Full Form
AMH	Anti-Müllerian Hormone
BAI	Body Adiposity Index
BMI	Body Mass Index
BMR	Basal Metabolic Rate
CRP	C-reactive protein
E2	Estradiol
EREs	Estrogen response elements
ECDs	Endocrine disrupting chemicals
ELISA	Enzyme-Linked Immunosorbent Assay
ESHRE	European Society for Human Reproduction and Embryology
ER	Estrogen receptors
FSH	Follicle Stimulating Hormone
GnRH	Gonadotropin-Releasing Hormone
GPER	G protein-coupled estrogen receptors
GJIC	Gap junction intercellular communication
HA	Hyperandrogenism
HPLC	High Performance Liquid Chromatography
HPO	Hypothalamic–Pituitary–Ovarian
IGFBPs	Insulin-like growth factor-binding proteins
IGF	Insulin-like growth factor
IR	Insulin Resistance
LH	Luteinizing Hormone
MAPKS	Mitogen-Activated Protein Kinase
NIH	National Institute of Health
OD	Ovulatory Dysfunction
PCOM	Polycystic Ovarian Morphology
PCOS	Polycystic Ovary Syndrome
PCBs	Polychlorinated biphenyls
PKA	Protein kinase A
PI3K	Phosphoinositide 3-kinase Signaling
PRO	Prolactin
RCAES	Revised Rotterdam Criteria of the Androgen Excess Society
ROS	Reactive oxygen species
SHBG	Sex Hormone Binding Globulin
T2DM	Type 2 Diabetes Mellitus
TLC	Thin Layer Chromatography
VAI	Visceral Adiposity Index
WC	Waist Circumference
WHO	World Health Organization
ZEN	Zearalenone

Summary

Polycystic Ovary Syndrome (PCOS) is a complex endocrine disorder characterized by sex hormone imbalances, leading to reproductive and metabolic dysfunction. Though the primary drivers of hormonal dysregulation in PCOS are well-studied, the roles of emerging factors like environmental endocrine disruptors and novel endogenous proteins in sex hormone homeostasis are gaining attention. Zearalenone, a mycoestrogen, is known for its estrogenic activity, and Kallistatin, an adipokine, has roles in angiogenesis, inflammation, and metabolic regulation. Their potential influence on the intricate hormonal milieu of PCOS remains to be fully elucidated.

This study aimed to investigate the levels of Zearalenone and Kallistatin in women with PCOS and to explore their association with key sex hormone parameters, thereby shedding light on their potential impact on sex hormone homeostasis in this patient population.

A cross-sectional study was conducted from September 2024 to July 2025 in Iraq, recruiting 138 women from the Obstetrics and Gynecology Hospital in Karbala and Baghdad Teaching Hospital (Medical City). Included 98 patients diagnosed with Polycystic Ovary Syndrome (PCOS) and 40 apparently healthy women who served as a control group. Data were collected via a structured questionnaire covering sociodemographics, medical history, and lifestyle, complemented by clinical evaluations by specialist physicians.

Ninety-eight women aged 18-44 years were diagnosed with PCOS based on the 2012 Rotterdam consensus criteria: presence of at least two of three features: clinical/biochemical hyperandrogenism, oligo- or amenorrhea, and/or polycystic ovarian morphology (PCOM) via ultrasonography. Clinical hyperandrogenism was assessed by hirsutism (modified Ferriman–Gallwey score), acne, or androgenic alopecia, though biochemical hyperandrogenism involved elevated serum androgens. Menstrual irregularities included oligomenorrhea (>35-day cycles) or amenorrhea (<4-6 cycles/year). PCOM was defined as ovarian volume >10 mL and/or ≥ 12 follicles (2-9 mm). Patients were categorized into four phenotypes: Phenotype A: Anovulation (A), Hyperandrogenism (HA), Polycystic Ovaries (PCO), Phenotype B: A, HA without PCOM, Phenotype C: HA, PCO with ovulatory cycle and Phenotype D: A, PCO without HA

Detailed demographic and clinical data were collected, including anthropometric measurements (body weight, height, waist/hip circumference for BMI, BAI, VAI, LAP, BMR calculation), menstrual patterns, fertility status, and PCOS-related symptoms. Blood samples were analyzed for lipid profiles and reproductive hormones (FSH, LH, testosterone, E2).

Participants were excluded if they were pregnant or breastfeeding, had known endocrine disorders (e.g., thyroid dysfunction, hyperprolactinemia, congenital adrenal hyperplasia), diabetes mellitus, cardiovascular diseases, hepatic/renal dysfunction, autoimmune diseases, or were receiving corticosteroid therapy. These criteria aimed to minimize confounding factors and ensure a focused study population specific to PCOS.

This study investigated the levels of the mycoestrogen Zearalenone and the adipokine Kallistatin, alongside sex hormones and metabolic indices, in 98 Iraqi women with Polycystic Ovary Syndrome (PCOS) across various phenotypes (Rotterdam A, B, C, D), infertility types, and menstrual cycle regularities, comparing them to 40 healthy controls.

Results revealed significant hormonal and metabolic distinctions among PCOS subgroups. Patients with irregular menstrual cycles and classic hyperandrogenic phenotypes (A and B) exhibited higher testosterone and lower sex hormone-binding globulin (SHBG), coupled with elevated visceral adiposity index (VAI) and lipid accumulation product (LAP), indicating a more severe metabolic and hormonal profile.

Kallistatin levels were consistently and significantly lower in PCOS patients, particularly in those with irregular menstrual cycles and the more severe classic phenotypes (A, B, C), compared to healthy controls. Conversely, Kallistatin levels were notably higher in Normo-androgenic PCOS (Phenotype D), approaching those of healthy controls. ROC curve analysis demonstrated Kallistatin's excellent discriminatory power for diagnosing Classic PCOS (Phenotype B: AUC 0.95; Phenotype A: AUC 0.88) and good power for Ovulatory PCOS (Phenotype C: AUC 0.83), but poor utility for Normo-androgenic PCOS (Phenotype D: AUC 0.64).

Zearalenone levels were generally similar or slightly elevated in PCOS patients versus controls, with the highest levels observed in the Un-married and irregular menstrual cycle groups, and notably in Normo-androgenic PCOS (Phenotype D). However, Zearalenone exhibited overall poor diagnostic discriminatory power across most phenotypes (AUCs 0.60-0.61 for A, B, C),

showing only a modest diagnostic ability for Normo-androgenic PCOS (Phenotype D: AUC 0.75).

In conclusion, Kallistatin appears to be a strong potential diagnostic and characterizing biomarker for hyperandrogenic PCOS phenotypes, reflecting metabolic and ovulatory health. Zearalenone's role is more nuanced, potentially acting as an environmental contributor in specific PCOS subgroups. These findings underscore the heterogeneous nature of PCOS and emphasize the importance of phenotype-specific biomarker evaluation for improved diagnosis and understanding of the disease.

CHAPTER ONE

Introduction



1. Introduction

Polycystic ovarian syndrome (PCOS) is the most common endocrine pathology in females of reproductive worldwide. Stein and Leventhal initially described it in 1935. The prevalence ranges between 5% and 26% (**Bozdag & et al, 2016**), depending on the diagnostic criteria applied. It is widely accepted among specialty society guidelines that the diagnosis of PCOS must be based on the presence of at least 2 of the following 3 criteria: chronic anovulation, hyperandrogenism (clinical or biological), and polycystic ovaries. PCOS is a diagnosis of exclusion, and disorders that mimic clinical features of PCOS must be excluded. Delays in diagnosis can lead to the progression of comorbidities, making it more difficult to implement lifestyle intervention, which is critical for the improvement of features of PCOS and quality of life. Polycystic ovarian syndrome it is considered the of the most prevalent endocrine and metabolic disorder in reproductive women, with a prevalence affecting 5–20%, with up to 70% of affected women remaining undiagnosed (**Pervin, 2022**).

It is characterized by hyperandrogenism, anovulatory cycles, the presence of multiple small follicles in one or both ovaries, infertility, and a higher risk of metabolic disorders such as; type 2 diabetes, liver fat disease, hypertension, clotting of blood vessels, possibly cardiovascular events, complications of childbirth, cancer, and possibly malignant tumors in the ovary and mood and psychological disorders (**Xu, 2022**) (**Ali, 2024**).

Primarily, it is affected by alteration of the hypothalamus–pituitary–ovarian (HPO) axis. The cyclic pattern of hormone concentrations, which is the characteristic determinant of normal ovulatory menstrual cycles, is lost in women with PCOS. In these women, there is chronic anovulation resulting in a “steady low state” of gonadotropin and sex steroid concentrations (**Ehrmann, 2005**).

The etiology of PCOS is likely complicated and involves environmental, genetic, metabolic, endocrine, and epigenetic factors. Women with PCOS have elevated LH-to-FSH ratios, low-normal follicle-stimulating hormone (FSH) levels, and higher levels of luteinizing hormone (LH) concentrations. Insulin resistance is prevalent in obese PCOS women and less common in lean PCOS women. The prevalence usually range between 50 and 75 percent. (**A., Dunaif, 1997**)

Up to 10% of women with polycystic ovaries meet the criteria for type 2 diabetes mellitus, and about 35% of those affected have impaired glucose tolerance. Insulin and LH act synergistically, resulting hyperandrogenism. Insulin resistance and hyperinsulinemia are the cause, not the result, of hyperandrogenism in PCOS.

Environmental and Endocrine Disruptors: Role of Zearalenone

Previous research has highlighted the role of environmental factors, including endocrine disrupting chemicals (EDCs), in the pathogenesis of PCOS. Among these factors, zearalenone (ZEN), a mycotoxin produced by *Fusarium* fungi, has attracted attention due to its estrogenic properties (**Zinedine et al., 2017**)

ZEN structurally mimics 17 β -estradiol and can bind to estrogen receptors (ER α and ER β), with agonist or antagonist effects depending on the tissue context (Tiemann, 2003) This mimicry disrupts normal estrogen signaling, potentially leading to reproductive toxicity. Studies in animal models have shown that ZEN exposure affects ovarian folliculogenesis, hormone secretion, and uterine morphology (**Jedrzejczak P. & Pawelczyk, 2015**)

The effect of ZEN on human reproductive health is an emerging area of research. Its ability to influence sex hormone balance suggests a potential role in the onset or exacerbation of PCOS, particularly given the chronic exposure to low levels of dietary exposure in humans (**Zinedine et al., 2017**). Understanding the effect of ZEN on hormone regulation may shed light on the contribution of environmental factors to the pathophysiology of PCOS.

Kallistatin: A Regulatory Protein in Ovarian Function

Kallistatin, encoded by the SERPINA4 gene, is a serine protease inhibitor with diverse biological activities, including anti-inflammatory, antioxidant, antifibrotic, and angiogenic effects (Chao, 2010). It has been extensively studied in cardiovascular and renal diseases, but its role in reproductive endocrinology is still in its infancy.

Emerging evidence suggests that Kallistatin may regulate ovarian function by modulating angiogenesis, oxidative stress, and inflammation in ovarian tissue (**Wang, 2019**). These processes are crucial for follicular development and ovulation, which are often disrupted in PCOS.

In addition, the interactions of Kallistatin with hormonal signaling pathways may affect sex hormone balance.

Investigations into kallistatin levels in PCOS patients have yielded preliminary data indicating altered expression, which could contribute to the syndrome's pathogenesis (Li, 2021). However, the mechanisms by which kallistatin affects sex hormone homeostasis remain to be fully elucidated.

1.1 Literature review

In 1935 were recognized Leventhal and Stein as the main researchers of PCOS, though Vallisneri, an Italian physician, naturalist, and medical scientist, defined PCOS in 1721 when she observed many infertile women as enlarged size ovaries as pigeon ovum size with a shining white surface. Formal diagnostic criteria were not presented until the early 1990s at a National Institute of Health (NIH) sponsored reaching on PCOS, and they were subsequently widely used (de Oliveira, 2020) .

Many scientists found to explain the pathophysiology of PCOS and many studies were made. It is currently believed that it is multifactorial, partly genetic; yet, a number of candidate genes have been hypothesized. Insulin resistance is frequently seen in numerous women with PCOS, especially those with hyperandrogenism; yet, it is missing from the diagnostic criteria. The criteria developed by a group of specialists during a conference in Rotterdam held on 2003 are obligatory (The Rotterdam ESHRE/ASRM - Sponsored PCOS Consensus Workshop Group). The more recent "Rotterdam criteria" incorporated the size and structure, as analyzed by an ultrasound, of the ovary into the diagnostic criteria. (Szydlarska, 2017)

1.2. Clinical Manifestations of Polycystic Ovarian Syndrome

A diverse condition marked by excessive levels of the hormone testosterone and chronic ovulatory failure. Elevated serum levels of luteinizing hormone (LH) and an irregular blood ratio of LH to follicle-stimulating hormone (FSH), which results in hyperandrogenism, are additional characteristics of PCOS females. (Iervolino, 2021)

Menstrual abnormalities and signs of an excess of testosterone are examples of clinical symptoms. (such as alopecia, acne, and hirsutism) (Teede & et al, 2018) as illustrated in Figure (1).

The endocrine and reproductive characteristics of PCOS can be developed by insulin resistance and hyperinsulinemia; nevertheless, hyperandrogenism may affect insulin action directly or through several mechanisms of change occurring in several body parts, including fat and muscle tissue (Moggetti & Tosi, 2020)

Polycystic ovary syndrome (PCOS) affects multiple body systems and its manifestations extend beyond reproductive problems. The main clinical symptoms are menstrual irregularities (oligomenorrhea or amenorrhea), infertility and symptoms associated with androgen excess (hirsutism, acne, seborrheic hirsutism and androgenic alopecia) (Azziz R. et al, 2016). In addition, many patients suffer from obesity or central obesity, which worsens metabolic dysfunction.

Women with PCOS are at increased risk for long-term complications, such as type 2 diabetes, hyperlipidemia, hypertension, and cardiovascular disease (Wild & et al, 2010). PCOS is also associated with an increased prevalence of psychiatric disorders, including anxiety and depression, complicating their treatment (Cooney, 2017).

Insulin resistance and hyperinsulinemia have the ability to develop endocrine and reproductive symptoms of PCOS, however, hyperandrogenemia can affect insulin action directly and / indirectly variations that take place in several parts of the body mostly muscle and adipose tissue (Moggetti & Tosi, 2020). Long-term metabolic syndrome development, including type II diabetes mellitus(T2DM) and cardiovascular disease, is most common in females with polycystic ovarian syndrome. (Dadachanji & Mukherjee, 2018)

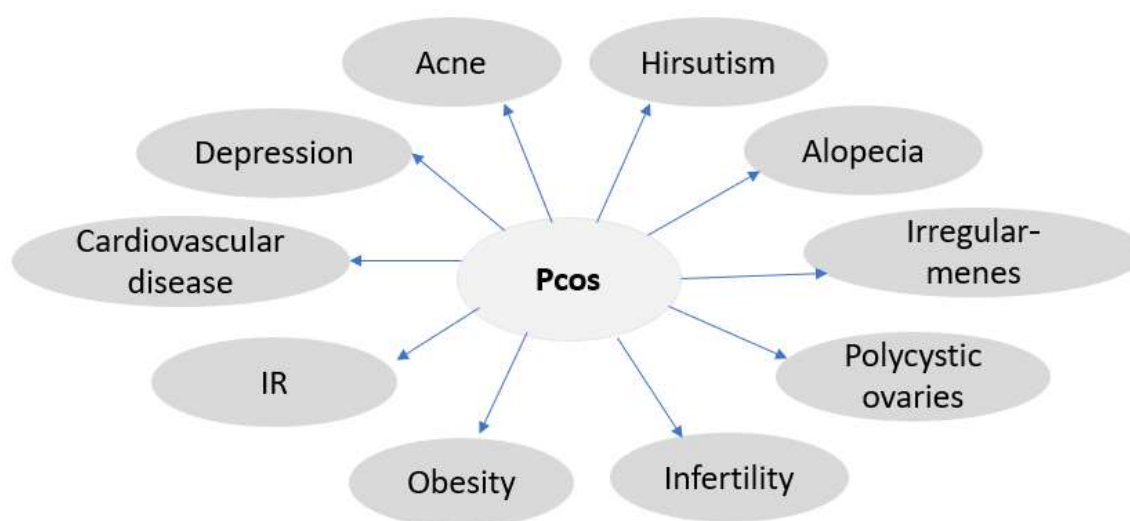


Figure (1-1): Clinical Manifestations (by author)

1.3. Etiopathogenesis of Polycystic Ovarian Syndrome

It has been reported that the etiopathogenesis of PCOS is a complex of genetic, environmental, and lifestyle interactions. The pathophysiology and neuroendocrine disturbances of the hypothalamic-pituitary-gonadal axis in PCOS can be explained by increased pulsatility of gonadotropin-releasing hormone (GnRH), leading to increased frequency of luteinizing

hormone (LH) secretion and decreased frequency of follicle-stimulating hormone (FSH), thereby disrupting folliculogenesis and increasing ovarian androgen production. Adrenal androgens are also increased, and androgen excess stimulates abdominal adipose tissue deposition, subsequently leading to increased insulin resistance and hyperinsulinemia. **(Fauser, 2023)**

Identifying this disorder as a syndrome is not a disease due to the presence of a number of symptoms that cannot be identified as a specific pathogen or a certified physiological axis. In addition, hormones are believed to move in most tissues of the body, but at a variable speed, this leads to a change in clinical features due to hormonal disorders and metabolic consequences. **(Krug & Paganini, 2019)**

The pathophysiology of PCOS is unknown, but substantial evidence suggests that it is a multifactorial disorder in which interactions between intrinsic endocrine, metabolic, genetic, and environmental factors work in harmony to produce a common outcome **(Yau, 2017)**

Worldwide, PCOS affects between 8% and 20% of women of reproductive age each year, according to diagnostic criteria **(Witchel & et al, 2019)**. The pathophysiology of this condition is influenced by alterations in steroidogenesis, ovarian folliculogenesis, neuroendocrine function, metabolism, insulin production, insulin sensitivity, adipose cell activity, inflammatory factors, and sympathetic nerve function **(Ibáñez & et al., 2017)**. According to Barre et al., high carbohydrate intake, hyperinsulinemia, hyperandrogenemia, and persistent low-grade inflammation are the four key factors that contribute to the pathophysiological alterations in PCOS **(Barrea & et al., 2018)**. (Figure 1-2)

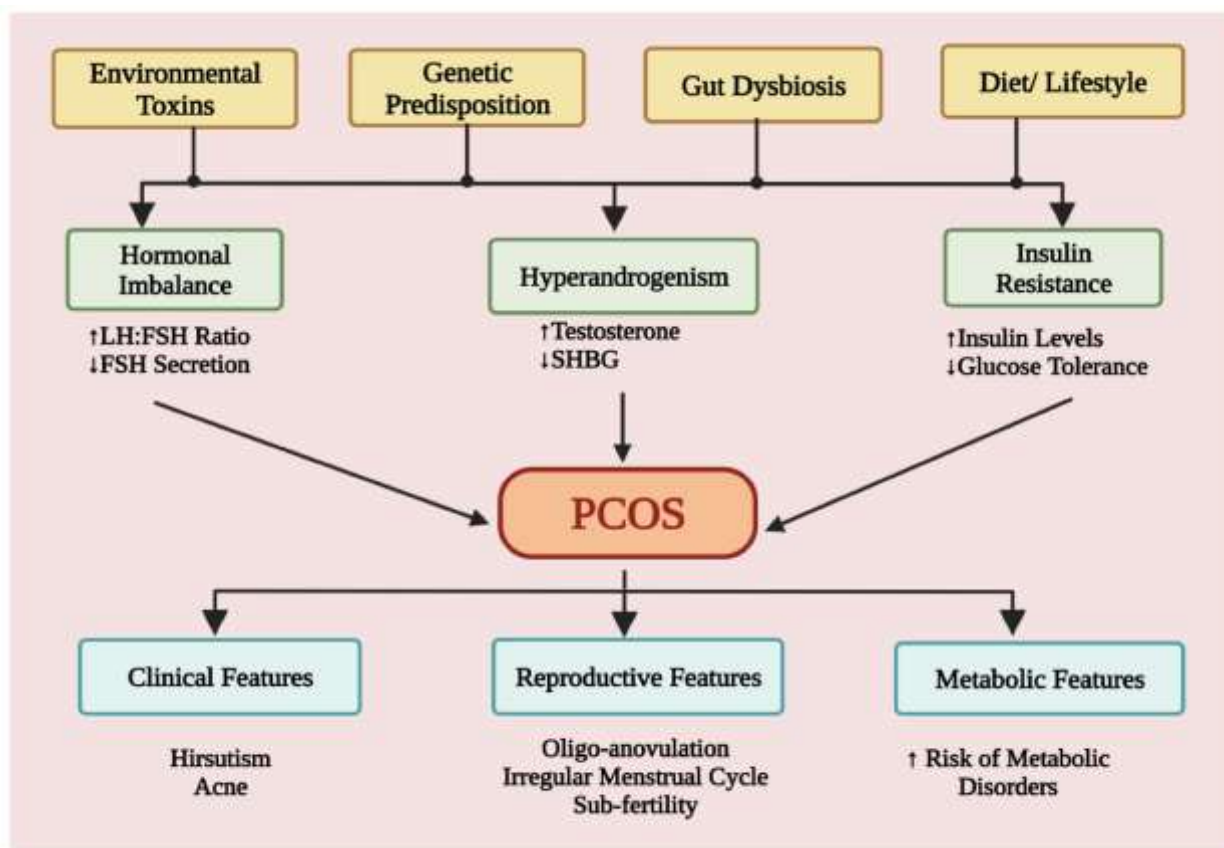


Figure (1-2). This schematic illustration shows the proposed pathophysiology and features of PCOS. The risk factors like environmental toxins, genetics, gut dysbiosis, and diet responsible for the pathophysiology of the PCOS and subsequent development of clinical, reproductive and metabolic features in PCOS patients. LH: luteinizing hormone; FSH: Follicle stimulating hormone; SHBG: Sex hormone binding globulin. (Barrea & et al., 2018)

1.4. Epidemiology of Polycystic ovary syndrome

With increasing evidence, PCOS is recognized worldwide as the most common endocrine disorder in women of reproductive age, affecting between 5% and 26% of women. The Rotterdam Criteria, the most widely used criteria in epidemiologic studies, include more inclusive criteria than the second most widely used criteria from the National Institutes of Health from 1990. The revised Rotterdam Criteria of the Androgen Excess Society (RCAES) are more specific in including the PCOS phenotype with polycystic ovarian morphology (PCOM) and infertility than the Rotterdam Criteria (Rasquin, 2022).

The rate of hirsutism was lower in women diagnosed according to the National Institutes of Health (NIH) criteria than in women diagnosed according to the RC and RCAES criteria. In

women diagnosed according to the RC criteria, the prevalence of insulin resistance (IR) was lower than in women diagnosed according to the NIH and RCAES criteria. **(Deswal R, Narwal V, Dang A, Pundir CS., 2020)**

In contrast, more than 80% of women with hyperandrogenism have polycystic ovary syndrome (PCOS). **(Sirmans SM, Pate KA., 2013)** According to a 2012 National Institutes of Health workshop report, PCOS is estimated to affect approximately 5 million women of reproductive age in the United States.

1.4.1 Prevalence of Polycystic Ovarian Syndrome

1.4.1.1 Global Prevalence of PCOS

Worldwide, the estimated prevalence of PCOS varies between 5% and 21%, depending on the diagnostic criteria used:

- National Institutes of Health criteria: approximately 5-10%
- Rotterdam criteria: approximately 12-21%
- AES criteria: intermediate

USA: ~6.6% (NIH criteria), ~15% (Rotterdam) **(Azziz et al., 2004)**

- China: ~5.6% (Rotterdam) **(Li et al., 2013)**
- India: ~8.2–22.5% (varies by region) **(Nidhi et al, 2011)**
- Europe: ~6–15% depending on country and criteria **(March et al, 2010)**

In a study among the Middle East and North Africa (MENA), the age-standardized prevalence and incidence of PCOS were 37.9% (95% CI: 31.7-45.0) and 33.7% (95% CI: 27.7-40.3) **(Motlagh Asghari et al, 2022)**

1.4.1.2 Prevalence of PCOS in the Middle East

In the Middle East, it is more common than the global average. This is often attributed to lifestyle factors such as obesity, physical inactivity, insulin resistance and genetic predisposition.

Country	Prevalence	Diagnostic Criteria	Study Type
Iran	14.6%	Rotterdam	Community-based (Tehrani, 2011)
Saudi Arabia	16%	Rotterdam	Hospital-based (El-Sherbiny et al, 2018)
UAE	13%	Rotterdam	Clinical population (Alnaqbi et al, 2020)
Kuwait	12.5%	Rotterdam	Cross-sectional (AlKandari et al, 2022)
Jordan	13.1%	Rotterdam	Community-based (Obeidat et al, 2010)

The average regional prevalence of the disease (Middle East) ranges from (12% to 18%).

1.4.1.3. Prevalence of PCOS in Iraq

There are no large-scale epidemiological studies in Iraq. Most data come from hospital-based studies, which often show higher prevalence rates than population studies.

- Al-Diwaniyah: 12–21% of reproductive-aged women attending gynecology clinics had PCOS; prevalence increased to 30% among obese women (Al-Magazchy & Sharif, 2023)
- Babylon: ~35% of women presenting with reproductive issues were diagnosed with PCOS, rising to 45% among married woman. (Mousa, B.A. & Makki Al-Joborae, S.F.F., 2020)
- Baghdad: 18.5% of women visit fertility clinics (Hussein et al., 2020)
- Kirkuk: 20.3% of women visit outpatient gynecological clinics (Ahmed & Ali, 2019)

The high prevalence may be influenced by:

- Lifestyle changes after the end of the conflict
- Poor access to early screening and health education
- Increasing obesity and sedentary behavior

Comparative Overview

Region	Prevalence Range	Notes
GLOBAL	5% _ 21%	Varies by criteria and ethnicity
middle east	12% _ 18%	High obesity/insulin resistance
Iraq	18.5% _ 20.3%	Based on hospital samples; higher than regional avg

The prevalence of polycystic ovary syndrome (PCOS) in Iraq appears to be higher than regional (Middle Eastern) and global averages. This may indicate a genuine public health concern or an increase in the number of cases due to sampling in clinical settings.

Prevalence was higher than in other Asian populations, reaching 28.9% according to the National Institutes of Health criteria, 35.3% according to the Rotterdam criteria, and 34.3% according to the criteria for symptomatic PCOS. These differences between studies using the same diagnostic criteria in different countries may be attributable to differences in race/ethnicity, geographic factors, and differences in study sample characteristics. **(Ganie et al, 2020)**

1.4.2. Incidence of PCOS

A data from the 2019 Global Burden of Disease Study indicate a significant increase in PCOS cases worldwide: the global age-standardized incidence rate was 59.8 per 100,000 women in 2019, representing a 29.5% increase since 1990. **(GBD 2019 Diseases and Injuries Collaborators, 2019)**. Specifically, the Middle East and North Africa region, including Iraq, recorded an incidence rate of 77.2 per 100,000 women in 2019, representing a 33.7% increase since 1990.

In contrast, Iraq lacks official data on population-level infection rates. Instead, hospital and clinic studies provide valuable information: A 2020 study in Babylon reported that 35% of women with menstrual or fertility problems were newly diagnosed with PCOS, an indicator of infection rates among symptomatic patients. **(Mousa, B.A. & Makki Al-Joborae, S.F.F., 2020)**. A sample of a fertility clinic in Baghdad between 2017 and 2018 showed an infection rate of around 45% among married women seeking treatment **(Hussein, S.A., Jasim, R.H. & Hussein, R.H., 2020)**. Though these figures are not directly comparable to regional norms, they suggest a high rate of clinical detection among symptomatic Iraqi women.

1.5. Hypothalamic–Pituitary–Ovarian Axis Alterations

In healthy females, the hypothalamic–pituitary–ovarian (HPO) axis maintains reproductive homeostasis through the pulsatile release of gonadotropin-releasing hormone (GnRH) from the hypothalamus. GnRH stimulates the anterior pituitary to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH). FSH promotes granulosa cell proliferation and estradiol synthesis, though LH acts on theca cells to produce androgens, which are subsequently aromatized into estrogens within granulosa cells **(Daan, et al., 2021)**

In polycystic ovary syndrome (PCOS), this tightly controlled axis becomes disrupted. Women with PCOS often exhibit increased GnRH pulse frequency, which preferentially promotes LH secretion over FSH. This leads to an elevated LH:FSH ratio, frequently exceeding 2:1 or 3:1, which contributes to excessive androgen production by theca cells and disrupts follicular development **(Rosenfield & Ehrmann, 2022)**. Simultaneously, the low FSH levels impair the selection of a dominant follicle, resulting in anovulation and accumulation of small, arrested follicles—observable as polycystic morphology on ultrasound. **(Zhou, Y. et al, 2023)**, as shown in figure (1-3), the hypothalamic–pituitary–ovarian axis behaves differently in PCOS patients compared to healthy individuals **(BioRender, n.d.)**.

Chronic LH hyperstimulation induces theca cell hyperplasia and increases follicular fluid accumulation, giving rise to the classic “string of pearls” appearance on the ovarian periphery. Arrested follicles are typically halted at the pre-antral or antral stage, and overexpression of steroidogenic enzymes such as CYP17A1 and CYP11A1 enhances androgen biosynthesis **(Dumesic, Lobo, & Abbott., 2022)**

Moreover, recent insights have highlighted the role of kisspeptin and GABAergic neuronal dysregulation in the hypothalamus. Kisspeptin neurons, part of the KNDy (kisspeptin–neurokinin B–dynorphin) system, are hypersensitive to sex steroids in PCOS, amplifying GnRH output. Concurrently, reduced GABA inhibition leads to enhanced GnRH neuronal firing, contributing to neuroendocrine abnormalities **(Kauffman, A.S. et al., 2020) (Moore, A.M. et al., 2023)**

Insulin resistance, a hallmark of PCOS, further exacerbates the condition by amplifying LH-stimulated androgen production in theca cells and impairing negative feedback of estrogen and Prolactin on the hypothalamus and pituitary **(Goodarzi et al., 2021)**. Additionally, low-grade inflammation and immune dysregulation have been implicated, with recent evidence showing

elevated autoantibodies against ovarian tissue and the GnRH receptor in a subset of PCOS patients (Zhao et al., 2024)

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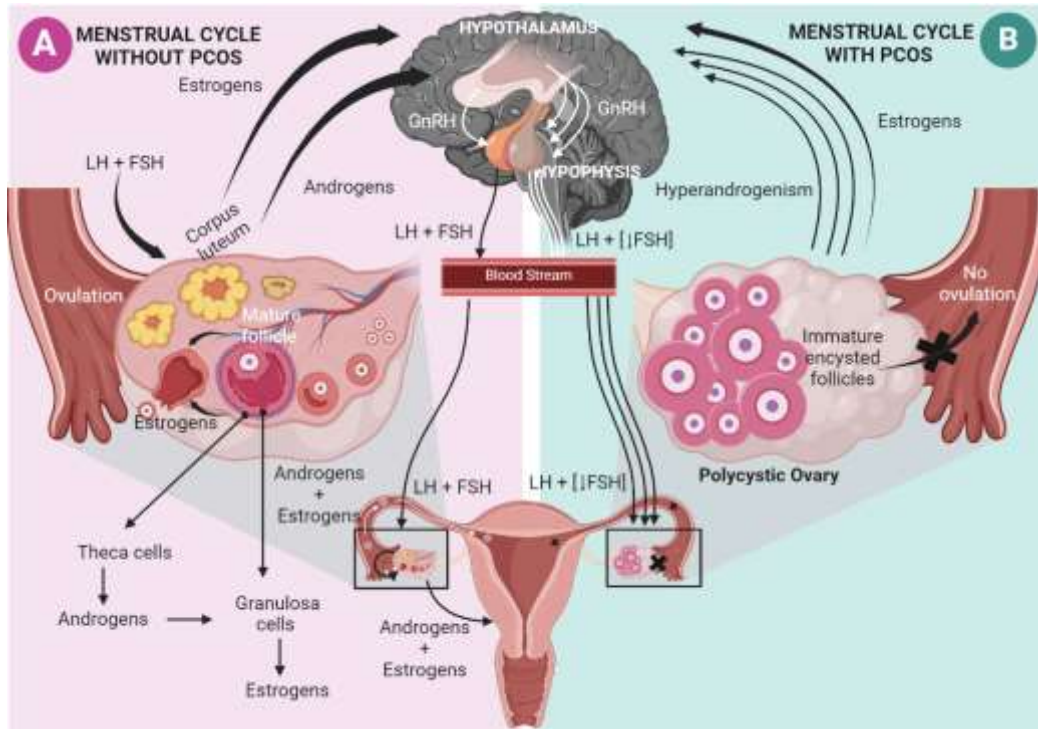


Figure (1-3) Hypothalamic–pituitary–ovarian axis behaviour in people with PCOS

Functioning of the hypothalamic-pituitary-ovarian axis during the menstrual cycle in people without PCOS (A), and in people with PCOS (B). (FSH, Follicle Stimulating Hormone. LH, Luteinizing Hormone. GnRH, Gonadotropin Releasing Hormone). (Zhao et al., 2024).

1.6. Diagnostic Criteria of Polycystic Ovarian Syndrome

PCOS can be diagnosed in any female when at least two of these three features: are clinical hyperandrogenic, ovulation abnormalities, and an ultrasound image showing cysts in the ovaries. This type of diagnosis is termed a Rotterdam Criteria for the diagnosis of PCOS (Kolhe, et al., 2022). For the diagnosis of PCOS in premenopausal women, there are three sets of diagnostic criteria for PCOS that have been recommended for use in identifying premenopausal women with the syndrome in table (1-1)

Table(1-1) :The Common Diagnostic Criteria for PCOS

Parameter	NIH 1990	ESHRE/ASRM 2003	AE-PCOS 2006	NIH 2012 extension of ESHRE/ASRM 2003
Criteria	➤ HA OD	➤ HA ➤ OD PCOM	➤ HA ➤ 2. OD and/or PCOM	➤ HA ➤ OD ➤ PCOM
Limitations	2 of 2 Criteria required	2 of 3 Criteria Required	2 of 2 Criteria Required	2 of 3 Criteria required; and identification of specific phenotypes: A: HA + OD + PCOM B: HA + OD C: HA + PCOM D: OD + PCOM

NIH= National Institutes of Health; ESHRE/ASRM= European Society for Human Reproduction and Embryology (ESHRE) and the American Society for Reproductive Medicine (ASRM); AE-PCOS= Androgen Excess and PCOS Society; HA= Hyperandrogenism; OD=ovarian dysfunction; PCOM= polycystic ovarian morphology (**Lizneva, et al., 2016**)

Updated Diagnostic Criteria for PCOS (2023 Guidelines):

The 2023 International Evidence-Based Guideline for the Assessment and Management of Polycystic Ovary Syndrome (PCOS) introduced a refined diagnostic framework for adult women. As per the updated recommendations, a diagnosis of PCOS should be established when

two of the following three features are present, provided that alternative causes with overlapping clinical features have been excluded:

- Clinical and/or biochemical signs of hyperandrogenism, which may present as hirsutism, acne, or elevated androgen concentrations in laboratory testing.
- Ovulatory dysfunction, commonly indicated by irregular or absent menstrual cycles due to infrequent or absent ovulation.
- Polycystic ovarian morphology is identified either through ultrasound findings or elevated levels of Anti-Müllerian Hormone (AMH), which may serve as a surrogate marker when imaging is inconclusive or unavailable.

This updated model emphasizes a more individualized and evidence-based approach to diagnosis, particularly highlighting AMH as a potentially useful diagnostic marker in clinical settings. **(Teede & et al ., 2023)**

1.6.1. Hyperandrogenism

Hyperandrogenism is a core pathological hallmark of PCOS and plays a key role in perpetuating both reproductive and metabolic disturbances. Excess androgens—especially testosterone, androstenedione, and the emerging class of C11-oxy androgens like 11-ketotestosterone—are primarily produced by dysregulated ovarian theca cells under the influence of increased luteinizing hormone (LH) and hyperinsulinemia **(O'Reilly, 2023); (Lemaitre, 2023)**

These androgens impair follicular development and contribute to anovulation, though also inducing clinical manifestations such as hirsutism and acne (Escobar-Morreale, 2023). Furthermore, they disrupt the hypothalamic–pituitary–ovarian axis by altering GnRH pulse frequency, further stimulating LH hypersecretion and creating a vicious cycle of androgen excess **(Review on PCOS Pathology, 2025).**

Additionally, hyperandrogenism worsens insulin resistance by promoting visceral adiposity and adipocyte dysfunction, reinforcing the endocrine-metabolic loop central to PCOS pathogenesis. Targeted therapies such as insulin sensitizers and anti-androgen agents aim to

disrupt this loop and improve both ovulatory and metabolic outcomes (**Spritzer, Lecke & Satler, 2015**).

1.6.2. Ovarian Dysfunction

Ovarian dysfunction represents a core component of Polycystic Ovary Syndrome (PCOS), contributing significantly to its reproductive and endocrine abnormalities. The main ovarian abnormality involves disrupted folliculogenesis, whereby follicle development is prematurely arrested. This follicular arrest leads to irregular or absent ovulation, which underlies many of the menstrual irregularities and infertility issues experienced by women with PCOS. (**Rosenfield, & Ehrmann, 2020**) (**Dumesic & Padmanabhan, 2020**)

This dysfunction is primarily driven by hormonal imbalances, notably elevated androgen levels and insulin resistance. Hyperandrogenism disrupts normal follicle maturation, causing the accumulation of numerous small, immature follicles characteristic of polycystic ovarian morphology observed on ultrasound scans (**Teede & et al, 2020**) (**Goodarzi & et al, 2021**)

Another significant factor is the elevated anti-Müllerian hormone (AMH) levels found in PCOS patients. AMH, produced by small growing follicles, may inhibit follicle sensitivity to FSH, thus contributing to follicular arrest and anovulation (**Rosenfield, 2021**). Consequently, AMH serves both as a useful diagnostic biomarker and a potential therapeutic target in PCOS management (**Teede & et al 2020**)

Structural ovarian alterations, such as increased ovarian volume and stromal hyperplasia, also accompany these hormonal disturbances, creating an environment that hinders normal follicular development (**Dumesic & Padmanabhan 2020**). Collectively, these morphological and endocrine changes explain the persistent ovulatory dysfunction and subfertility characteristic of PCOS.

In summary, ovarian dysfunction in PCOS results from a multifaceted interplay of hormonal imbalances and structural ovarian changes that impair folliculogenesis and ovulation. Gaining a comprehensive understanding of these mechanisms is vital for

developing targeted treatments to restore ovulatory function and improve fertility outcomes in affected women.

1.6.3. Polycystic Ovarian Morphology

Polycystic ovarian morphology is a key diagnostic marker that refers to the ultrasonographic appearance of the ovaries. Historically, PCOM was defined by the presence of ≥ 12 follicles measuring 2–9 mm in diameter and/or an ovarian volume $>10 \text{ cm}^3$ in at least one ovary. However, with advances in ultrasound technology, particularly with high-frequency transducers ($\geq 8 \text{ MHz}$), the 2018 international guidelines recommend increasing the follicle count threshold to ≥ 20 follicles per ovary though maintaining the same volume criterion (**Teede & et al, 2018**); (**Dewailly, 2014**).

It is important to note that PCOM alone is not pathognomonic for PCOS, as a similar ovarian appearance may be observed in healthy individuals, especially adolescents and women with irregular cycles during the perimenarchal period (**Tay & et al, 2020**). Therefore, its diagnostic value increases significantly when assessed alongside ovulatory and androgenic parameters. Furthermore, reliance on ultrasound quality and technique introduces variability in PCOM detection, which is why consistent imaging protocols and operator training are essential for accurate diagnosis (**Broekmans & et al, 2020**).

Though PCOM enhances the diagnostic sensitivity of PCOS, particularly in non-hyperandrogenic phenotypes, its role should be interpreted cautiously, especially in younger patients. Contemporary research emphasizes the need for phenotype-specific management strategies and a nuanced understanding of ovarian morphology to avoid overdiagnosis and unnecessary interventions (**Tay & et al, 2020**); (**Peña & et al, 2022**).

1.7. Phenotypes of Polycystic Ovarian Syndrome.

Polycystic Ovary Syndrome (PCOS) presents in various phenotypic forms, reflecting differences in clinical, endocrine, and metabolic features among affected individuals. The widely accepted Rotterdam criteria classify PCOS into four phenotypes, based on the presence

or absence of three key features: hyperandrogenism (HA), ovulatory dysfunction (OD), and polycystic ovarian morphology (PCOM).

These phenotypes are defined as follows:

- Phenotype A (HA + OD + PCOM)
- Phenotype B (HA + OD)
- Phenotype C (HA + PCOM)
- Phenotype D (OD + PCOM)

Phenotype A, often referred to as the “classic” form, is the most prevalent and is typically associated with more severe endocrine and metabolic abnormalities (**Tehrani & et al, 2022**) (**Livadas, S. & Diamanti-Kandarakis, E, 2013**)

Recent studies have demonstrated significant heterogeneity in metabolic risk across these phenotypes. Women exhibiting phenotypes A, B, or C, all of which include hyperandrogenism, tend to show increased insulin resistance, elevated triglycerides, and a greater prevalence of metabolic syndrome compared to those with phenotype D, who lack biochemical or clinical evidence of androgen excess (**Tehrani & et al, 2022**); (**Murumoglu, S. and Yildiz, B.O., 2020**). In contrast, phenotype D, though marked by ovulatory dysfunction and polycystic ovarian morphology, often demonstrates a milder clinical course and relatively favorable metabolic profiles, particularly in older women (**Rees & et al, 2022**).

Moreover, phenotype-specific differences have been observed in reproductive outcomes. For instance, phenotypes A and D have been independently associated with increased risk of pregnancy complications during assisted reproductive technology procedures, highlighting the clinical relevance of phenotypic classification in fertility management (**Wang & et al, 2022**). In addition, emerging evidence suggests that phenotype D may represent a distinct subset within the PCOS spectrum, potentially linked to unique immunological or endocrine mechanisms, separate from the androgen-excess-driven pathophysiology seen in other phenotypes (**Rees & et al, 2022**).

Taken together, the classification of PCOS into phenotypes offers a practical and clinically meaningful approach that aids in tailoring management strategies. Recognizing these subtypes helps clinicians to differentiate patients who require metabolic intervention from those who primarily present with reproductive challenges. As research continues to evolve, a deeper

understanding of the underlying mechanisms distinguishing these phenotypes may lead to more targeted and effective therapeutic approaches.as in figure (1_4).

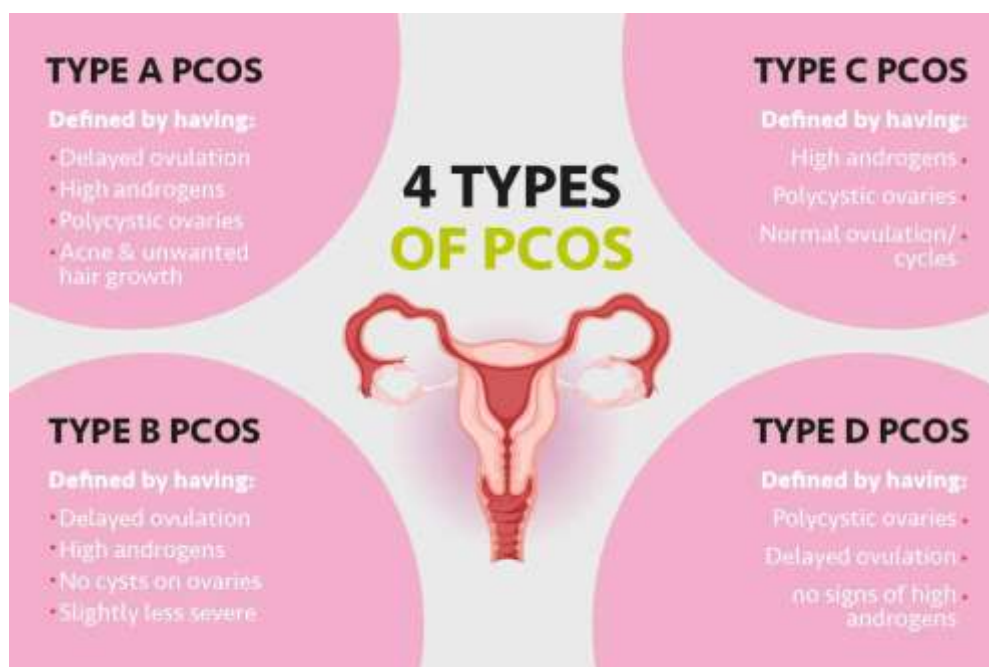


Figure (1-4). The four phenotypes of PCOS based on the Rotterdam criteria: Type A (HA + OD + PCOM), Type B (HA + OD), Type C (HA + PCOM), and Type D (OD + PCOM). (OSH Wellness, n.d.)

1.8. Risk Factors of Polycystic Ovarian Syndrome

Polycystic ovary syndrome (PCOS) is attributed to a multifactorial etiology involving genetic, metabolic, and environmental factors. Genetic factors have been consistently documented, with familial clustering suggesting a genetic predisposition to the disease. The main modifiable risk factors are insulin resistance and obesity, both of which contribute to the development and worsening of PCOS by increasing hormonal imbalance and disrupting ovulatory function (Osibogun, O., Ogunmoroti, O. and Michos, E.D., , 2020). In addition, a sedentary lifestyle, poor dietary habits, and early puberty are considered contributing environmental and lifestyle factors. Identifying and understanding these risk factors are essential for early intervention and the implementation of effective preventive strategies.

Women diagnosed with polycystic ovary syndrome (PCOS) face an elevated risk of various pregnancy-related complications, such as gestational diabetes mellitus, hypertensive disorders like pre-eclampsia, abnormal fetal growth including macrosomia and small-for-

gestational-age outcomes, as well as increased perinatal mortality. These risks demonstrate that the impact of PCOS extends far beyond reproductive dysfunction, with consequences that span a woman's entire lifespan. Many of the associated health concerns are chronic in nature and may require long-term management (**Bahri Khomami & et al, 2019**)

1.8.1. Insulin Resistance

Insulin resistance (IR) is a key feature of polycystic ovary syndrome (PCOS), affecting 50–70% of women, independent of body mass index (**Webb, 2021**). It impairs cellular glucose uptake, prompting compensatory hyperinsulinemia that enhances ovarian androgen production and contributes to reproductive dysfunction, including anovulation and hyperandrogenism (**Zhou et al., 2023; Moghetti & Bonin, 2023**). Obesity further aggravates IR, with over 80% of overweight/obese women with PCOS showing impaired insulin sensitivity (**Wang et al., 2022**).

Acanthosis nigricans (AN) serves as a clinical marker of IR in PCOS, driven by hyperinsulinemia-mediated IGF-1 receptor activation leading to epidermal proliferation (**Leung et al., 2023; Radu et al., 2022**).

At the molecular level, IR in PCOS involves increased serine and reduced tyrosine phosphorylation of insulin receptor substrates, disrupting the PI3K/Akt pathway in skeletal muscle, adipose tissue, and ovarian cells (**Zhao et al., 2023**). Reduced insulin receptor β -subunit expression in visceral fat and diminished GLUT4 in subcutaneous tissue also hinder glucose uptake (**Miller et al., 2022**). Mitochondrial dysfunction, MAPK–ERK overactivation, and possible CNS-related defects further contribute to systemic IR (**Zhao et al., 2023; Miller et al., 2022**). Interestingly, insulin signaling in ovarian steroidogenesis remains intact, selectively stimulating androgen synthesis via MAPK-related pathways (**Nguyen et al., 2023; Corbould et al., 2006**).

Insulin promotes hyperandrogenism by enhancing cytochrome P450c17 α activity and synergizing with LH on theca cells (**Morgante et al., 2020; Diamanti-Kandarakis & Dunaif, 2012**). It also disrupts follicular maturation, limiting growth to 5–10 mm and impairing ovulation (**Kumariya et al., 2021**). As a co-gonadotropin, insulin augments LH effects by stimulating ovarian insulin/IGF receptors and amplifying GnRH-driven LH

secretion, while lowering hepatic SHBG production, thereby increasing free androgen levels (Siemienowicz et al., 2021).

Hyperinsulinemia also reduces IGFBPs, raising IGF-I bioavailability, which amplifies ovarian androgen production and LH secretion, perpetuating hyperandrogenism and anovulation (Firmansyah et al., 2018; Turnbull & Williams, 2021). Furthermore, androgens exacerbate IR by altering muscle structure and promoting ectopic fat deposition, creating a vicious cycle of metabolic and reproductive dysfunction (Escobar-Morreale, H.F., 2023) (Chazenbalk & et al, 2022), as in figure (1-5).

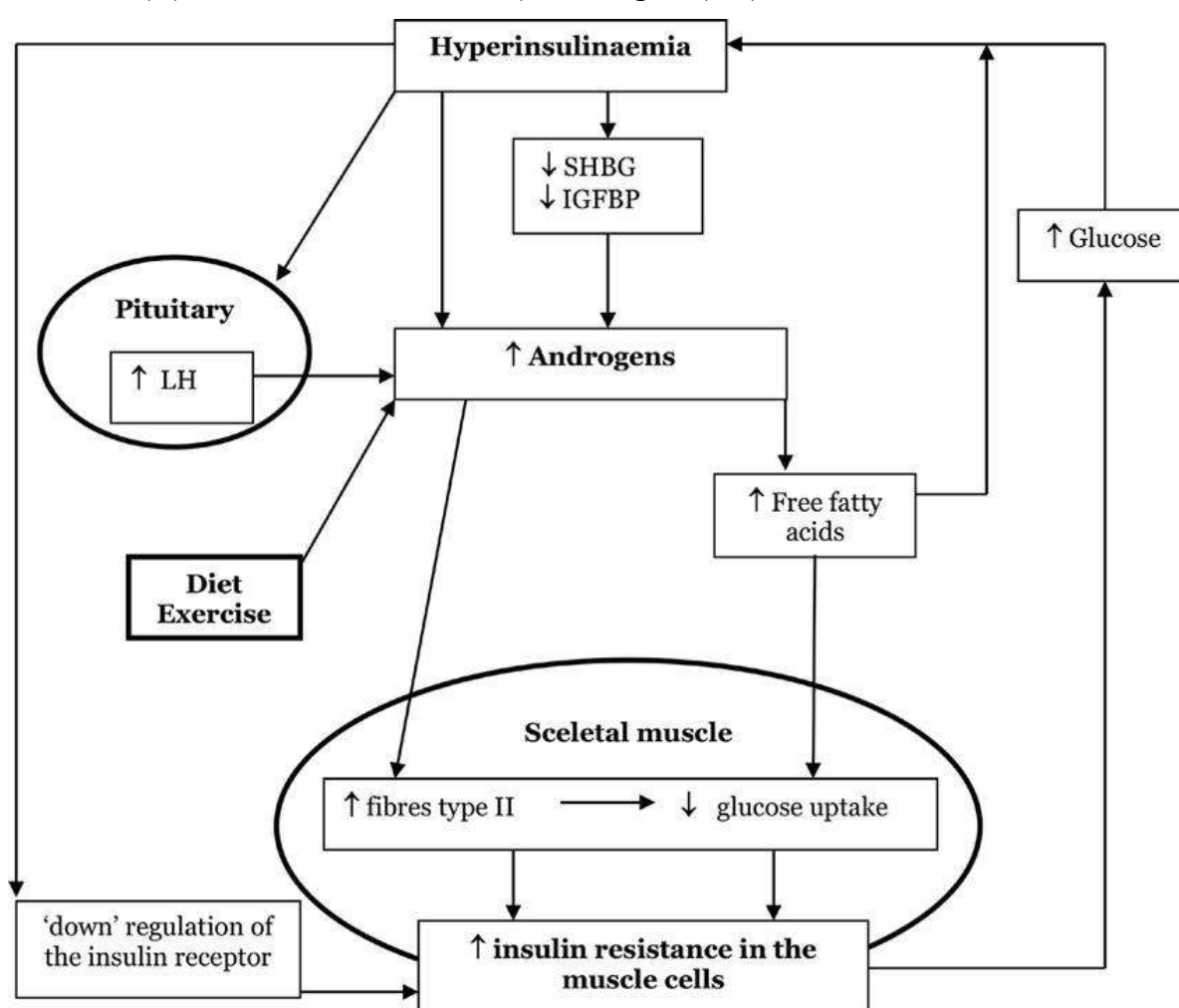


Figure (1_5): Role of insulin in the pathogenesis of PCOS (Kamenov and Gateva, 2020)

1.8.2. Obesity

Obesity—particularly central or visceral adiposity—is a critical contributor to the worsening of both reproductive and metabolic features of PCOS. Visceral fat is metabolically active, releasing pro-inflammatory cytokines and free fatty acids, which

directly impair insulin signaling in skeletal muscle, liver, and ovarian tissue **(Kalra, B. et al., 2009); (Jia, Y. et al, 2023)**. Women with PCOS also exhibit a disproportionate accumulation of abdominal fat at a given BMI, indicating a disrupted energy regulatory system **(Kalra, B. et al., 2009); (Escobar-Morreale, H.F., 2023)**

Obese PCOS patients typically exhibit higher insulin resistance and elevated androgen levels compared to their lean peers, contributing to menstrual irregularities, hirsutism, and anovulation **(Wang,Y.et al, 2022) (Escobar-Morreale, 2023)**. Moreover, childhood adiposity—such as early adiposity rebound—has been linked to an increased adult PCOS risk, emphasizing the importance of early weight management **(Koivuaho, E. et al., 2019)**. Collectively, this evidence positions central obesity as both a driver and amplifier of PCOS pathology, underscoring the need for targeted prevention and therapeutic interventions.

Importantly, this global pattern is reinforced by multiple Iraqi studies. For instance, Kasim and Saadoon (2022) reported that 60% of women with PCOS in Iraq were classified as obese ($\text{BMI} \geq 30 \text{ kg/m}^2$), in contrast to nearly none among healthy controls, with obesity correlating significantly to elevated LH and testosterone **(Kasim, S.F. and Saadoon, S.J., 2022)**. A study from Baghdad confirmed a strong association between central obesity and adverse hormonal profiles in PCOS **(Al-Gareeb, A.I. et al, 2015)** Additionally, a research in Duhok province highlighted that between 30–75% of women with PCOS exhibited obesity, further supporting the link between adiposity and PCOS severity **(Rasool, 2021)** These regional findings mirror global evidence and highlight the critical need for culturally adapted weight management interventions to mitigate PCOS progression in Iraq.

1.8.3. Genetic Predisposition

Genetic predisposition plays a crucial role in the development of polycystic ovary syndrome (PCOS), highlighting the importance of inherited susceptibility in its pathogenesis. Evidence from familial clustering and twin studies has consistently shown that first-degree relatives of women with PCOS are at higher risk of developing the condition themselves, suggesting a strong heritable component **(Goodarzi & al, 2011)**. Several candidate genes related to insulin signaling, androgen biosynthesis, and gonadotropin action have been investigated, although the complex nature of PCOS makes it unlikely that a single gene is responsible. Instead, PCOS is now regarded as a polygenic

disorder, where multiple gene variants interact with environmental and hormonal factors to influence disease expression **(Li & et al, 2022)**

Genome-wide association studies (GWAS) have identified numerous loci associated with PCOS, including genes such as DENND1A, THADA, and FSHB, which are thought to impact hormonal regulation and metabolic functions **(Day & et al ., 2018)** These genetic markers have been observed across diverse populations, although their expression and penetrance may vary by ethnicity. This supports the hypothesis that genetic predisposition interacts with lifestyle and environmental factors to determine the onset and severity of PCOS features.

Though genetic factors do not solely cause PCOS, their role in predisposing individuals to the syndrome—especially when coupled with poor lifestyle habits or obesity—emphasizes the multifactorial nature of this disorder. Understanding these inherited risks may help guide early diagnosis and personalized interventions, particularly in individuals with a family history of PCOS.

1.8.4. Lifestyle Factors and PCOS

Lifestyle patterns play a significant role in the development and progression of polycystic ovary syndrome (PCOS), particularly through their influence on metabolic health, hormonal regulation, and weight management. Sedentary behavior, poor dietary choices, and insufficient physical activity have all been implicated in worsening insulin resistance and obesity, two core features of PCOS **(Moran & et al, 2011) (Teede, 2023)**. High intake of processed carbohydrates and saturated fats is associated with elevated serum insulin levels and impaired glucose metabolism, thereby promoting hyperandrogenism and ovulatory dysfunction **(Barrea & et al, 2022)**.

Conversely, studies have demonstrated that lifestyle modification, including structured exercise and dietary adjustments, significantly improves insulin sensitivity, menstrual regularity, and fertility outcomes in women with PCOS **(Teede & et al, 2018)**. Even modest weight loss (as little as 5–10%) has been shown to reduce serum androgen levels and restore ovulatory cycles **(Moran & et al, 2011)**. Furthermore, behavioral patterns such as chronic stress, irregular sleep, and poor time management may contribute to hormonal imbalance

by disrupting cortisol levels and circadian rhythm, indirectly exacerbating PCOS features (Bhattacharya, S. and Jha, A, 2020).

Given the modifiable nature of these factors, early lifestyle interventions represent a primary strategy for both the prevention and management of PCOS, especially in high-risk groups.

1.8.5. Environmental Factors and PCOS

In recent years, the role of environmental exposures in the development of polycystic ovary syndrome (PCOS) has gained increasing attention, particularly due to the influence of endocrine-disrupting chemicals (EDCs) on hormonal regulation and metabolic homeostasis. These compounds—including bisphenol A (BPA), phthalates, and polychlorinated biphenyls (PCBs)—are prevalent in many consumer products, such as plastic containers, cosmetics, agricultural chemicals, and industrial emissions. Due to their ability to either mimic endogenous estrogens or interfere with androgen metabolism, these substances may initiate or intensify symptoms associated with PCOS (Kandaraki E. et al, 2011) (Wang L. et al, 2021)

Among the various EDCs, BPA has emerged as a compound of particular concern, given its widespread use and its measurable presence in human biological samples. Elevated levels of BPA have been consistently observed in women diagnosed with PCOS when compared to healthy controls. These elevations have been associated with hormonal disturbances such as increased serum testosterone, insulin resistance, and abnormal folliculogenesis, all of which are central features of PCOS pathology (Kandaraki E. et al, 2011) (Zhang Y. et al, 2023).

Beyond chemical exposures, other environmental stressors—including poor air quality, climate-related changes, and the pressures of densely populated urban living—have also been linked to compromised reproductive health. These factors may influence PCOS risk through mechanisms involving systemic oxidative stress and chronic low-grade inflammation. Furthermore, such environmental burdens may act in conjunction with genetic predispositions and lifestyle behaviors, thereby amplifying the susceptibility to or severity of the syndrome (Zhou, 2020) (Rattan & et al, 2022) .

Considering these insights, it becomes essential to take into account the environmental setting in which individuals live when assessing PCOS risk and formulating prevention or

management strategies. This is particularly important in urbanized and industrial regions, where exposure to multiple risk factors is often unavoidable.

Overall, PCOS appears to result from the interplay of several contributing elements, including inherited tendencies, obesity, lack of physical activity, and environmental stressors. These components act not only as initial triggers but also as accelerators of the syndrome's progression, further complicating its reproductive and metabolic manifestations. Understanding the complex interactions among these variables is fundamental to capturing the full scope of PCOS pathophysiology and tailoring more effective approaches for early intervention and clinical care.

1.9. Complications and Long-Term Health Consequences of PCOS

Polycystic Ovary Syndrome (PCOS) is not only a reproductive disorder but a chronic systemic condition that carries far-reaching health implications across multiple domains, especially when left unmanaged. The progression of PCOS often evolves from irregular ovulatory function and hyperandrogenism in early reproductive years to metabolic and cardiovascular complications in later life.

1.9.1. Metabolic Complications

One of the most concerning long-term effects of PCOS is the development of insulin resistance and hyperinsulinemia, which occur in up to 70% of affected women (**Teede et al, 2023**). Over time, persistent insulin resistance increases the risk of type 2 diabetes mellitus (T2DM), with studies indicating that women with PCOS have a 2- to 4-fold higher risk of developing T2DM compared to women without the condition. Furthermore, metabolic syndrome—which includes abdominal obesity, dyslipidemia, hypertension, and glucose intolerance—is highly prevalent in this population and is a key mediator of cardiovascular disease (**Lim et al, 2019**).

1.9.2. Cardiovascular Risks

PCOS is independently associated with several cardiovascular risk factors. These include elevated triglycerides, low HDL cholesterol, high blood pressure, and increased markers of inflammation such as C-reactive protein (CRP) (**Glntborg, D. and Andersen, M., 2021**). Though the direct link between PCOS and cardiovascular events like myocardial infarction remains under investigation, surrogate markers such as endothelial dysfunction and

increased carotid intima-media thickness suggest heightened vascular risk (**Sirmans, S.M. and Pate, K.A., 2014**).

1.9.3. Reproductive and Gynecological Complications

Infertility due to chronic anovulation is among the most distressing complications for women with PCOS. Even with ovulation induction therapies, the risk of pregnancy-related complications remains elevated. These include miscarriage, preterm birth, gestational diabetes, and preeclampsia (**Palomba et al, 2015**). In addition, due to unopposed estrogen exposure from irregular cycles, women are at risk of endometrial hyperplasia and, in some cases, endometrial carcinoma (**Barry et al, 2021**).

1.9.4. Psychological and Emotional Effects

The chronic nature of PCOS, especially when accompanied by symptoms like hirsutism, obesity, and infertility, can significantly affect mental well-being. Women with PCOS are more prone to depression, anxiety, body image dissatisfaction, and even eating disorders (**Alur-Gupta et al, 2021**). Psychological stress, in turn, can worsen hormonal imbalances, creating a self-perpetuating cycle.

1.9.5. Long-Term Outlook

When untreated or poorly managed, PCOS can evolve into a lifelong condition that compromises quality of life. As such, clinical guidelines now emphasize early diagnosis, comprehensive lifestyle interventions, and multidisciplinary care strategies to minimize the risk of long-term complications (**Teede et al, 2018**).

1.10. Overview of Fungi and Their Role in Mycotoxin Production

Fungi represent one of the most ancient and ecologically adaptable groups of eukaryotic organisms, forming a separate kingdom within the domain Eukarya. It is estimated that the total number of fungal species lies between 2.2 and 3.8 million, although only a fraction of these has been formally described to date (**Hawksworth, D.L. and Lücking, R., 2017**). These organisms are heterotrophic by nature, lacking chlorophyll and the ability to photosynthesize. Instead, they obtain nutrients by secreting enzymes into their surroundings and absorbing the resulting breakdown products, which allows them to act as

decomposers, pathogens, or symbiotic partners depending on the context (**Alexopoulos et al, 1996**).

A particularly important aspect of fungi in the context of public health is their capacity to produce mycotoxins—low molecular weight, biologically active secondary metabolites. Genera such as *Fusarium*, *Aspergillus*, and *Penicillium* are especially known for this ability. Mycotoxins are often synthesized in response to stress conditions and serve ecological purposes such as deterring predators or outcompeting other microbes (**Bennett and Klich, 2003**). Unfortunately, these same compounds can contaminate food crops, especially cereals, nuts, and legumes. Their chemical stability enables them to persist through storage and processing, retaining their toxic effects well beyond harvest. Key mycotoxins of concern include aflatoxins, fumonisins, ochratoxins, trichothecenes, and zearalenone (ZEN), all of which are associated with a wide range of toxic effects, including liver and kidney damage, immune dysfunction, and endocrine disruption (**Pitt J.I. and Hocking, 2009**) (**Zinedine et al, 2007**).

Understanding how fungi produce these toxins—and under what conditions—is essential for mitigating contamination and assessing their broader implications for food safety and human health.

1.10.1 Mycotoxins

Mycotoxins are toxic secondary metabolites produced mainly by *Aspergillus*, *Penicillium*, and *Fusarium* species under environmental stress conditions such as drought, high humidity, or nutrient limitation, often contaminating crops during cultivation or storage (**Bennett and Klich, 2003**). They belong to diverse chemical classes, including polyketides, amino acid derivatives, and terpenoids, and are grouped into major categories such as aflatoxins, ochratoxins, fumonisins, trichothecenes, and zearalenone, each with distinct toxicological effects (**Pitt and Hocking, 2009**). For instance, aflatoxins are hepatocarcinogenic, ochratoxin A is nephrotoxic and immunosuppressive, trichothecenes are cytotoxic, and fumonisins have been linked to esophageal cancer and neural tube defects (**Wild and Gong, 2010; Voss et al., 2007**).

Beyond acute toxicity, chronic low-level exposure has been associated with cancer, immune dysfunction, neurological disorders, and hormonal imbalances (**Khlangwiset et al., 2011**). Their chemical stability makes removal during food processing difficult,

posing particular risks in developing countries where regulation and control measures are limited (Eskola et al., 2020).

In addition to health impacts, mycotoxins cause major economic losses by reducing crop yield, animal productivity, and trade opportunities. Consequently, international organizations such as FAO, WHO, and the Codex Alimentarius Commission have set regulatory limits, but effective control still requires integrated pre-harvest, storage, and monitoring strategies.

The chemical diversity, environmental persistence, and broad toxicological profiles of mycotoxins underscore their enduring threat to food security and public health. Though these compounds serve as ecological tools for fungal survival and competition, their harmful impacts on human and animal health reveal a stark contradiction. This duality highlights the critical need for sustained interdisciplinary research in the field of mycotoxicology to better understand, detect, and mitigate their risks. See figure below

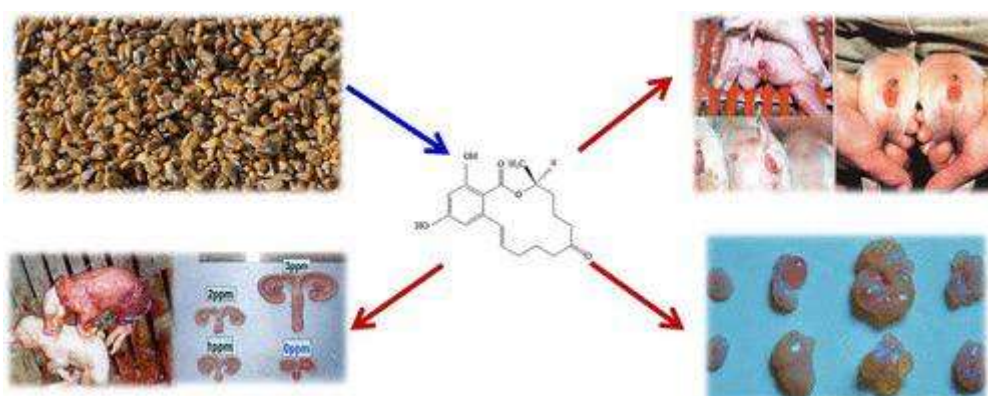


Figure (1-6) Feedstuffs are severely contaminated by zearalenone (ZEN) worldwide (Zinedine, 2007)

1.10.2. Zearalenone (ZEN): Structure, Origin, and Biological Properties

1.10.2.1 Origin and Chemical Nature

Zearalenone (ZEN) is a non-steroidal mycotoxin with potent estrogenic activity, primarily synthesized by *Fusarium* species such as *F. graminearum* and *F. culmorum*. It belongs to the resorcylic acid lactone group and is structurally defined by a macrocyclic 14-membered lactone ring featuring two hydroxyl groups and a keto group. This molecular configuration

(C₁₈H₂₂O₅) bears a strong resemblance to endogenous estrogens, particularly 17 β -estradiol. Due to this structural similarity, ZEN is capable of binding to estrogen receptors in mammalian systems, thereby exerting hormone-like biological effects (**Mirocha et al, 1976**); (**Borzekowski, 2018**) , see figure below .

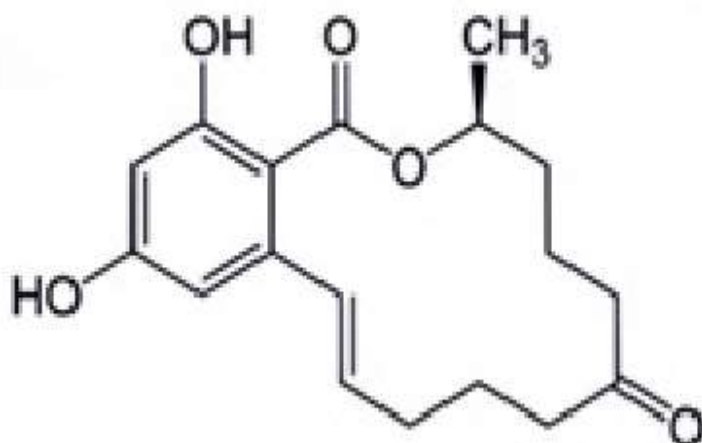


Figure (1-7) illustrates the molecular structure of ZEN, as described by Viñas and Karlovsky (2025) (**Viñas, 2025**)

1.10.2.2. Biosynthesis of ZEN

The biosynthesis of zearalenone (ZEN) follows a specialized polyketide pathway orchestrated by a defined cluster of genes. At the core of this process are two key polyketide synthase genes—PKS4 and PKS13 (also referred to as ZEA1 and ZEA2)—which function cooperatively to assemble the ZEN backbone. Biosynthesis begins with PKS4, catalyzing the condensation of acetyl-CoA and malonyl-CoA to produce a linear polyketide intermediate. This intermediate is subsequently elongated and cyclized by PKS13, forming the characteristic resorcylic acid lactone ring that defines ZEN's chemical structure (**Kim I.-K., 2005**) (**Gaffoor I. & Trail, 2006**). Further enzymatic modifications are mediated by ZEB1, an oxidoreductase responsible for introducing ketone functionalities, thus converting the intermediate into the biologically active form of ZEN (**Lysoe E. et al., 2006**).

ZEN production is tightly regulated at the transcriptional level. The ZEB2 gene encodes a bZIP-type transcription factor that governs the expression of the biosynthetic gene cluster. This regulation is modulated by signaling cascades, particularly the cAMP-dependent protein kinase A (PKA) pathway (**Lysoe E. et al., 2006**) (**Gaffoor I. & Trail, 2006**). Environmental stress conditions—such as changes in temperature, moisture, and nutrient

levels—can activate this regulatory system, promoting ZEN biosynthesis during *Fusarium* colonization of cereal crops.

1.10.2.3. Biological Properties and Ecological Role

Ecologically, zearalenone (ZEN) plays a strategic role in the survival and competitiveness of *Fusarium* species in their natural habitats. By acting as a chemical weapon, it enhances fungal dominance over other microorganisms, particularly under stressful environmental conditions. ZEN is frequently detected in cereal grains such as maize, wheat, and barley—especially when crops are cultivated or stored in humid environments conducive to fungal proliferation. One of the critical concerns surrounding ZEN is its high chemical stability, which enables it to endure food processing and storage conditions without significant degradation. This resilience allows the toxin to remain biologically active throughout the food supply chain, posing sustained risks to food safety and consumer health (**Borzekowski et al, 2018**) (**Eskola et al, 2020**)

From a biological standpoint, ZEN exerts its toxic effects primarily through its estrogen-mimicking activity. By binding to estrogen receptors, ZEN can interfere with normal endocrine signaling, leading to disruptions in reproductive development and function in both animals and humans. Such endocrine disruption is particularly concerning in populations with high dietary exposure to contaminated grains. The chronic nature of this exposure—often at subclinical levels—amplifies the toxin’s public health impact, reinforcing the urgent need for robust monitoring systems and preventive measures across agricultural and food production sectors.

1.10.3. Absorption, Metabolism, and Bioactivity of Zearalenone (ZEN)

Following oral ingestion, zearalenone (ZEN) is readily absorbed in the upper gastrointestinal tract, particularly within the proximal segments of the small intestine. Its lipophilic nature facilitates passive diffusion across intestinal membranes, enabling efficient uptake into systemic circulation (**Zinedine et al., 2007; EFSA, 2011**). Once in the bloodstream, ZEN undergoes hepatic biotransformation through enzymatic reduction, primarily by 3 α - and 3 β -hydroxysteroid dehydrogenases, which convert it into two major metabolites: α -zearalenol (α -ZEL) and β -zearalenol (β -ZEL) (**Gajęcka et al, 2011**).

These metabolites differ in their biological potency, with α -ZEL demonstrating a significantly higher affinity for estrogen receptors—particularly ER α —than either the

parent compound or β -ZEL. This enhanced receptor binding translates into a more pronounced activation of estrogenic signaling pathways in hormone-sensitive tissues, including the uterus, ovaries, and mammary glands **(Kuiper G.G. et al., 1998) (Pfeiffer E. et al., 2009)**. Moreover, both ZEN and its metabolites may undergo enterohepatic recirculation, a process that extends their systemic presence and biological activity **(EFSA, 2011) (Gajęcka et al., 2011)**.

Of additional concern is their ability to cross key physiological barriers such as the placental and blood-brain barriers. This characteristic raises the possibility of in utero exposure and central neuroendocrine disruption, potentially influencing developmental and reproductive outcomes **(Zinedine et al., 2007)**. The detection of ZEN and its metabolites in various tissues underscores their potential to induce wide-ranging endocrine disturbances, which have been implicated in conditions such as reproductive dysfunction, precocious puberty, and hormone-dependent cancers **(Guerre, 2015) (Zhang, 2020)**.

1.10.4. Mechanism of Action of ZEN and Its Metabolites

Zearalenone (ZEN), a non-steroidal estrogenic mycotoxin mainly produced by *Fusarium* species, exerts its biological effects by structurally mimicking 17β -estradiol, allowing it to interact with estrogen receptors ($ER\alpha$ and $ER\beta$) in target tissues. Although its binding affinity is lower than that of endogenous estrogens, ZEN and its metabolites can still activate or inhibit estrogenic pathways, thereby modulating the transcription of estrogen-responsive genes and disrupting endocrine balance **(Kuiper G.G. et al., 1998); (Zinedine A. et al., 2007)**. After ingestion, ZEN is absorbed in the gastrointestinal tract and undergoes hepatic metabolism to form α -zearalenol (α -ZEL) and β -zearalenol (β -ZEL), with α -ZEL demonstrating higher estrogenic potency due to its increased receptor affinity **(Borzekowski et al., 2018)**.

The interaction of ZEN and its metabolites with estrogen receptors leads to the formation of hormone-receptor complexes that translocate to the nucleus and bind to estrogen response elements (EREs) on DNA. This binding alters gene expression patterns involved in reproductive development, cell proliferation, and differentiation, especially in estrogen-sensitive tissues such as the uterus, mammary glands, and ovaries **(Shier, 2001); (Pfeiffer E. et al., 2009)**. Furthermore, these compounds can influence the hypothalamic-pituitary-

gonadal (HPG) axis, potentially disrupting gonadotropin release and ovarian steroidogenesis.

A recent research also suggests that ZEN can induce non-genomic effects through membrane-bound estrogen receptors or G protein-coupled estrogen receptors (GPER), activating rapid intracellular signaling pathways such as MAPK and PI3K/AKT cascades, which are associated with oxidative stress, inflammation, and carcinogenesis **(Guerre, 2015) (Zhang, 2020)**. Additionally, ZEN-induced oxidative stress and mitochondrial damage contribute to cytotoxicity and apoptosis, further supporting its classification as a potent endocrine-disrupting chemical.

Thus, the biological activity of ZEN is multifaceted, involving both genomic and non-genomic mechanisms. These mechanisms underlie a broad range of toxicological effects, particularly impacting reproductive physiology, and may contribute to long-term health risks, including hormone-dependent cancers and fertility impairments. See figure below

Zearalenone (ZEA) has been shown to exert dual cellular effects, promoting either proliferation or apoptosis depending on cellular context and exposure level. Its estrogenic activity arises from its ability to bind to estrogen receptors, thus mimicking endogenous estrogens and promoting the proliferation of hormone-sensitive cells. In addition to estrogen receptor-mediated actions, ZEA may induce tumorigenesis by damaging DNA and disturbing the function of gap junction intercellular communication (GJIC). In contrast, ZEA can also induce apoptotic pathways, involving the activation of endoplasmic reticulum (ER) stress responses,

generation of reactive oxygen species (ROS), and disruption of mitochondrial signaling cascades, ultimately leading to programmed cell death.

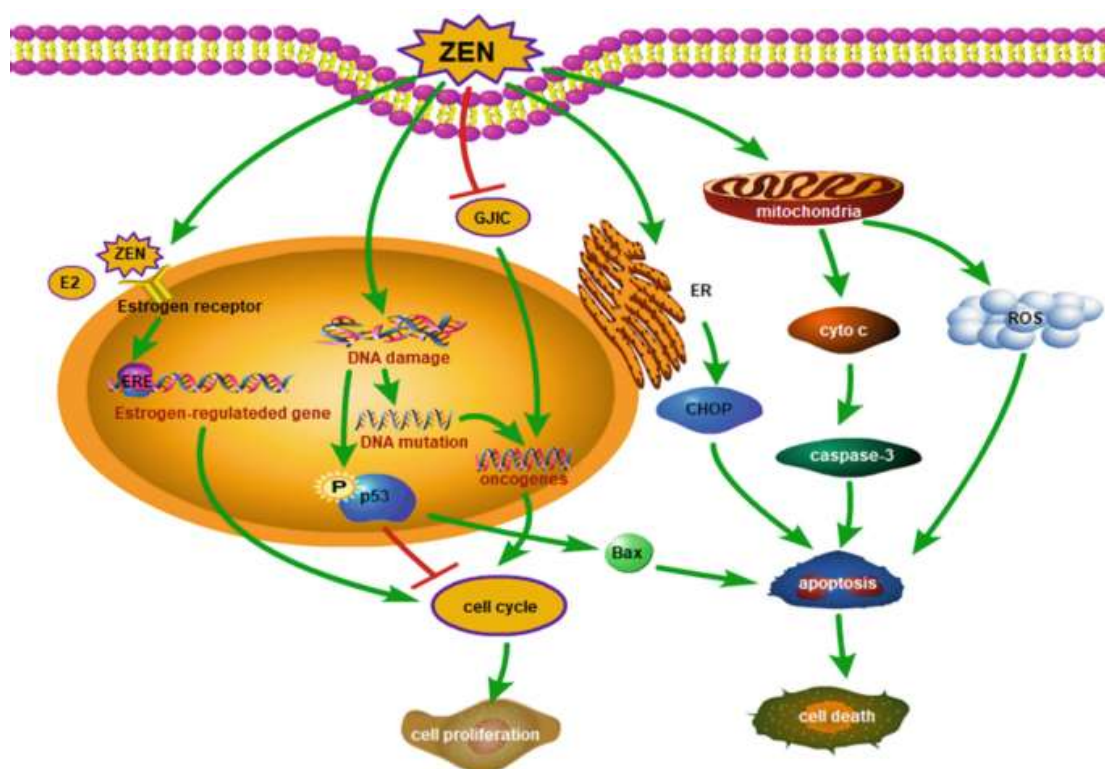


Figure (1-8). Molecular Mechanisms of Zearalenone (ZEA) in Cell Proliferation and Apoptosis. (PeerJ., 2020).

1.10.5. Estrogenic and Toxicological Effects of ZEN

Zearalenone (ZEN), a non-steroidal estrogenic mycotoxin mainly produced by *Fusarium* species, exerts its biological effects by structurally resembling 17β -estradiol, which enables it to bind to estrogen receptors ($ER\alpha$ and $ER\beta$) in target tissues. Although its affinity is lower than that of endogenous estrogens, ZEN and its metabolites can still activate or inhibit estrogenic signaling pathways. This interaction leads to altered transcription of estrogen-responsive genes and disruption of hormonal balance (Kuiper G.G. et al., 1998) (Zinedine A. et al., 2007). After ingestion, ZEN is absorbed in the gastrointestinal tract and undergoes hepatic metabolism, primarily forming α -zearalenol (α -ZEL) and β -zearalenol (β -ZEL), with α -ZEL showing greater estrogenic activity due to its higher receptor affinity (Borzekowski, 2018).

Once bound to estrogen receptors, ZEN and its metabolites form complexes that translocate to the nucleus, where they bind to estrogen response elements (EREs) on DNA. This alters

the expression of genes involved in reproductive development, cellular proliferation, and differentiation—particularly in estrogen-sensitive tissues such as the uterus, mammary glands, and ovaries (**Shier, 2001**) (**Pfeiffer E.et al, 2009**). In addition, these compounds can affect the hypothalamic-pituitary-gonadal (HPG) axis, disrupting gonadotropin secretion and ovarian steroidogenesis.

More recent findings suggest that ZEN can also trigger non-genomic effects via membrane-bound estrogen receptors or G protein-coupled estrogen receptors (GPER). These interactions activate rapid intracellular signaling cascades, such as MAPK and PI3K/AKT pathways, which are associated with oxidative stress, inflammation, and even cancer development (**Guerre, 2015**) (**Zhang, 2020**). Furthermore, ZEN-induced oxidative stress and mitochondrial dysfunction can promote cytotoxicity and programmed cell death, reinforcing its classification as a potent endocrine-disrupting compound.

In summary, ZEN's biological actions involve both genomic and non-genomic mechanisms, contributing to a broad spectrum of toxic effects. These include disturbances in reproductive function and potential long-term health risks, such as hormone-related cancers and infertility.

1.10.6. ZEN and Female Reproductive Health: A Pathophysiological Perspective

ZEN's interference with estrogenic pathways makes it particularly harmful to female reproductive health. In animal studies, exposure to ZEN has been shown to impair folliculogenesis, induce ovarian atrophy, and cause luteal dysfunction (**Shier, 2001**) (**Kim I.-K., 2005**). These effects are often mediated through the dysregulation of estrogen-responsive genes, resulting in abnormal development and functioning of reproductive tissues.

In granulosa cells, ZEN has been found to induce apoptosis via mitochondrial pathways and increase the production of reactive oxygen species (ROS), ultimately compromising oocyte quality and leading to ovulation failure (**Zhang, 2020**). Additionally, disruptions in the secretion of gonadotropins, including luteinizing hormone (LH) and follicle-stimulating hormone (FSH), have been observed, implicating ZEN in the dysregulation of the hypothalamic-pituitary-gonadal (HPG) axis (**Pfeiffer E.et al, 2009**) (**Guerre, 2015**).

Notably, both epidemiological observations and experimental models suggest a potential link between ZEN exposure and the development of reproductive disorders such as

endometriosis, precocious puberty, and infertility. Although direct evidence in humans remains limited, similarities with other endocrine-disrupting chemicals underscore the urgent need for further investigation, especially among vulnerable populations such as adolescent girls and pregnant women (Eskola et al, 2020) (Borzekowski, 2018).

Taken together, the pathophysiological effects induced by ZEN underline its serious threat to reproductive function and long-term fertility, supporting its classification as a reproductive toxicant of concern.

Building upon the established estrogenic and toxicological effects of zearalenone (ZEN) on female reproductive health, it is essential to explore its specific role in the pathophysiology of Polycystic Ovary Syndrome (PCOS), a common endocrine disorder marked by complex hormonal and metabolic imbalances.

1.10.7. The Role of Zearalenone (ZEN) in the Pathophysiology of Polycystic Ovary Syndrome (PCOS)

Zearalenone (ZEN) is a fungal-derived mycoestrogen primarily produced by *Fusarium* species, widely recognized for its ability to interfere with endocrine function by mimicking the action of endogenous estrogens. Due to its structural similarity to 17 β -estradiol, ZEN can bind to estrogen receptors (ER α and ER β), thereby influencing the hypothalamic-pituitary-gonadal (HPG) axis and altering normal patterns of gonadotropin secretion. This hormonal disruption is particularly relevant in the context of Polycystic Ovary Syndrome (PCOS)—a multifactorial disorder characterized by hyperandrogenism, anovulation, and polycystic ovarian morphology—where abnormal estrogen signaling and insulin resistance play central roles (Rashid, 2021) (Zhao, 2022).

A Mechanistic research indicates that ZEN exposure disrupts folliculogenesis by impairing granulosa cell function and the expression of key steroidogenic enzymes, potentially triggering oxidative stress and apoptosis. These cellular effects interfere with normal

estrogen synthesis and follicular development, contributing to the anovulatory cycles commonly seen in PCOS (Wang, 2023) (Li, 2021).

Additionally, ZEN has been shown to worsen insulin resistance by interfering with insulin signaling mechanisms, further exacerbating the metabolic abnormalities frequently associated with PCOS (Chen, 2020).

Animal studies involving chronic ZEN exposure have revealed PCOS-like features, including the formation of ovarian cysts and disruptions in the estrous cycle, reinforcing the compound's relevance to PCOS pathophysiology (Li, 2021). Considering that cereals and grains are often contaminated with ZEN and that the toxin remains stable during food processing, prolonged dietary exposure may pose a significant environmental risk factor contributing to the increasing prevalence of PCOS, especially in areas with high mycotoxin burden (Eskola et al, 2020) (Liu, 2022).

1.11. Kallistatin and Its Potential Role in Polycystic Ovary Syndrome (PCOS)

Kallistatin, encoded by the SERPINA4 gene, is a member of the serine protease inhibitor (serpin) family. It was initially identified as a natural inhibitor of tissue kallikrein but has since been recognized as a multifunctional protein involved in the regulation of inflammation, oxidative stress, angiogenesis, and vascular tone (Chao J. et al., 2016). Though predominantly synthesized in the liver, kallistatin is also expressed in the vasculature, kidneys, and reproductive organs, where it contributes to maintaining cellular homeostasis under physiological and pathological conditions (Chao & Chao, 2012)

Recent studies have highlighted kallistatin's relevance in metabolic and endocrine disorders, particularly those characterized by chronic low-grade inflammation and insulin resistance—two hallmarks of polycystic ovary syndrome (PCOS). PCOS is a heterogeneous condition that affects reproductive and metabolic health, often manifesting as hyperandrogenism, anovulation, and polycystic ovarian morphology. In this context, kallistatin's anti-inflammatory and anti-oxidant actions could play a protective role. For instance, it has been shown to inhibit pro-inflammatory cytokines such as TNF- α and IL-6, as well as reduce oxidative damage, which are both elevated in women with PCOS (Huang X. et al, 2020) (Yang Z. et al, 2021).

Moreover, kallistatin may influence ovarian function by modulating local angiogenesis and fibrosis—two processes known to be dysregulated in polycystic ovaries. Excessive stromal fibrosis and abnormal blood vessel formation are associated with poor follicular

development and hormonal imbalance. By counteracting these alterations, kallistatin may support normal folliculogenesis and hormonal regulation **(Wang Y. et al., 2019)**.

In terms of metabolic regulation, kallistatin has been linked to improved insulin sensitivity and vascular function. Given the high prevalence of insulin resistance in PCOS, reduced kallistatin levels may exacerbate metabolic disturbances, contributing to hyperinsulinemia and the subsequent overstimulation of androgen production in ovarian theca cells **(Liu, 2023)**.

1.12. Importance of Study and Knowledge Gap

Research Gaps in the Interplay Between Zearalenone and Kallistatin in PCOS: A Hormonal and Environmental Perspective was summarized by the following statement.

Zearalenone (ZEA), a fungal-derived mycoestrogen mainly synthesized by *Fusarium* species, has been widely studied in animal models for its endocrine-disrupting and reproductive toxic effects. Findings from rodent studies consistently demonstrate that prolonged exposure to low levels of ZEA leads to marked disturbances in hormonal balance, including increased serum concentrations of insulin, glucose, testosterone, Prolactin, and luteinizing hormone (LH), coupled with reductions in estradiol. These hormonal imbalances are often associated with ovarian morphological features closely resembling those seen in Polycystic Ovary Syndrome (PCOS), such as anovulation and follicular arrest (**Liu & et al, 2022**). However, despite substantial preclinical evidence, clinical investigations into circulating ZEA levels among women diagnosed with PCOS remain notably absent. Furthermore, the potential link between ZEA exposure and major clinical hallmarks of PCOS—such as insulin resistance, elevated androgens, and disrupted gonadotropin dynamics—has not yet been clarified in human studies (**Wang & et al, 2023**) (**Rashid & et al, 2021**).

In parallel, kallistatin—a member of the serpin (serine protease inhibitor) superfamily—has emerged as a molecule of growing interest due to its anti-inflammatory, antioxidative, and vasodilatory properties. Though kallistatin has been shown to influence vascular and immune-related pathways, its involvement in reproductive hormone regulation, especially within the hormonal dysregulation context of PCOS, is poorly understood. Preliminary data suggest a potential regulatory role for kallistatin in endocrine homeostasis; however, its specific associations with reproductive hormones such as LH, FSH, estradiol, and testosterone in PCOS-affected individuals have yet to be systematically investigated (**Chen & et al., 2020**) (**Li & et al., 2021**).

Importantly, the potential biological interaction between environmental estrogens like ZEA and endogenous regulators such as kallistatin in the pathogenesis of PCOS remains largely unstudied. The cellular and molecular mechanisms by which these two agents may converge to disrupt the hypothalamic-pituitary-ovarian (HPO) axis are still undefined.

Likewise, no existing clinical or experimental research has assessed whether targeting kallistatin expression or minimizing exposure to estrogen-mimicking toxins like ZEA could improve hormonal profiles or clinical outcomes in PCOS **(Zhao & et al, 2022) (Eskola & et al, 2020)**

This study seeks to address a part of major gaps by exploring the dual influence of environmental exposures and endogenous regulatory factors on combination with altered kallistatin level, might contribute to the hormonal disturbances characteristic of PCOS. This integrative approach may offer an insights into the underlying mechanisms of the syndrome.

1.13. Aims and Objectives of the study

- To Monitor and screen the Zearalenone levels in PCOS among Kerbala and Baghdad Females were the main objectives of the study
- To investigate the direct effects of Zearalenone on sex hormone production and regulation in PCOS patients with different phenotypes.
- To assess the correlation between Zearalenone exposure and the severity of PCOS symptoms, such as hirsutism, acne, and menstrual irregularities.
- To investigate the association between kallistatin levels and the severity of PCOS.
- To explore the role of kallistatin in the development and progression of hormonal disorder in PCOS.

CHAPTER TWO

Subjects, Materials and Methods



2.1. Study design

This study employed a cross-sectional design and was conducted over a three-month period, from December 2024 to February 2025. Participants were recruited from two main healthcare institutions: the Obstetrics and Gynecology Hospital in Karbala and the Consultation Clinic at Baghdad Teaching Hospital (Medical City), Iraq. A portion of the participants were enrolled through outpatient clinics at these centers. The study population included 138 women, comprising patients diagnosed with polycystic ovary syndrome (PCOS) and apparently healthy women who served as a comparison group.

Data were collected at a single point in time using a structured questionnaire that captured sociodemographic information, medical history, and lifestyle-related details. All participants underwent clinical evaluations conducted by board-certified specialist physicians to ensure accurate diagnosis and to rule out other possible conditions.

In addition to the questionnaire and clinical assessment, venous blood samples were collected for laboratory analysis. Biochemical investigations included serum lipid profile and key reproductive hormones such as follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone, and estradiol (E2), were performed.

Ethical Approval:

The study was approved by the Ethical Committee of the College of Medicine, University of Kerbala No: 24-59 Date:10/7/2025 .

2.1.1 Inclusion Criteria

Participants enrolled in this study were women aged between 18 and 44 years who fulfilled the diagnostic criteria for polycystic ovary syndrome (PCOS) according to the 2012 Rotterdam consensus. A diagnosis of PCOS was established when at least two out of the following three features were present: (1) clinical and/or biochemical hyperandrogenism, (2) oligo- or amenorrhea, and (3) polycystic ovarian morphology (PCOM) as confirmed by pelvic ultrasonography.

Clinical hyperandrogenism was identified through signs such as hirsutism, acne, or androgenic alopecia, with hirsutism evaluated using the modified Ferriman–Gallwey scoring system. Biochemical hyperandrogenism was defined by elevated serum androgen

concentrations. Menstrual irregularities included oligomenorrhea (cycles >35 days) or amenorrhea (fewer than four to six cycles per year). PCOM was characterized by an ovarian volume greater than 10 mL and/or the presence of fewer than 12 follicles measuring 2–9 mm in diameter.

Based on these diagnostic parameters, patients were further categorized into four PCOS phenotypes:

- Phenotype A: chronic anovulation, hyperandrogenism, and polycystic ovaries;
- Phenotype B: chronic anovulation and hyperandrogenism, without PCOM;
- Phenotype C: hyperandrogenism and PCOM, with preserved ovulatory cycles;
- Phenotype D: chronic anovulation and PCOM, in the absence of hyperandrogenism (Freeman, et al., 2022).

2.1.2. Exclusion Criteria

Participants were excluded from the study if they had conditions that could interfere with hormonal or metabolic evaluation. These included pregnancy or breastfeeding, known endocrine disorders such as thyroid dysfunction, hyperprolactinemia, or congenital adrenal hyperplasia. Women with a history of diabetes mellitus, cardiovascular diseases, hepatic or renal dysfunction, or autoimmune diseases were also excluded. Additionally, individuals receiving corticosteroid therapy at the time of recruitment were not considered eligible for participation. These exclusion criteria were applied to reduce potential confounding factors and to ensure a more homogeneous study population, allowing for a clearer assessment of the clinical and biochemical features associated specifically with polycystic ovary syndrome.

2.2. Subjects

2.2.1. Patients

This study involved 98 women who were diagnosed with polycystic ovary syndrome (PCOS) based on recognized diagnostic guidelines. Participants were collected over the period from December 2024 to February 2025 at two healthcare institutions: the Obstetrics and Gynecology Hospital in Karbala and the outpatient clinic at Baghdad Teaching Hospital (Medical City), Iraq.

Information about each participant was collected through a carefully designed questionnaire that covered a range of demographic and clinical factors. These included age,

body weight and height, waist and hip measurements, menstrual cycle patterns, fertility status and type of infertility when applicable, as well as symptoms commonly associated with PCOS such as excessive hair growth (hirsutism), acne, weight changes, and hair thinning or loss.

Clinical evaluations involved assessing hirsutism and calculating body composition indices, specifically body adiposity index (BAI), visceral adiposity index (VAI), body mass index (BMI), lipid accumulation product (LAP), and basal metabolic rate (BMR). Blood samples were also collected to measure lipid profiles and reproductive hormones, including follicle-stimulating hormone (FSH), luteinizing hormone (LH), testosterone, and estradiol (E2).

2.2.2. Control Group

A total of 40 apparently healthy females, aged between 18 and 44 years, were included as the control group. All participants had regular menstrual cycles and showed no clinical signs of polycystic ovary syndrome. Pelvic examination and ultrasound confirmed the absence of polycystic ovarian morphology.

Information was collected using the same structured questionnaire applied to the PCOS group, covering age, weight, height, waist and hip circumference, menstrual regularity, fertility status, and presence or absence of hirsutism.

Controls were selected based on the absence of any endocrine, metabolic, or chronic systemic conditions. Participants with a history of thyroid dysfunction, liver or kidney disease, diabetes mellitus (type 1 or type 2), cancer, stroke, autoimmune or inflammatory disorders such as rheumatoid arthritis were excluded, in order to ensure a metabolically and hormonally healthy comparison group.

2.2.3. Blood Collection and Storage

Venous blood samples were collected from all participants under standardized and sterile conditions to minimize pre-analytical variability. A volume of approximately 5 mL of blood was drawn from each subject using disposable syringes. For female participants in both the PCOS and control groups, blood collection was scheduled during the early follicular phase of the menstrual cycle, specifically between day 2 and day 5, to ensure hormonal consistency and improve the reliability of endocrine-related measurements.

The collected blood was allowed to clot at room temperature before centrifugation. Samples were centrifuged at 5000 rpm for 10 minutes to ensure complete separation of the serum.

The resulting serum was then carefully transferred into clean, 3 labeled Eppendorf tubes. All serum samples were promptly stored at -20°C until the time of biochemical analysis to preserve the integrity of sensitive analytes such as kallistatin and Zearalenone (ZEA). To prevent degradation, samples were handled cautiously and protected from repeated freeze

thaw cycles. A unique identification code was assigned to each sample to ensure proper documentation, traceability, and blinding during laboratory assessments.

2.2.4. Materials

Table (2-1): Materials and Instruments used in the study.

NO	Apparatus	Manufactures
1	Autoclave device	Lap Tech, Korea
2	Beaker	Volac, England
3	Candle Jar	China Mheco , China
4	Cap + Septa	Newstar
5	Capillary tube	vitrex , China
6	Centrifuge	HUMAN Humax4K , Germany
7	Deep Freeze	Royal ,England
8	Disposable Syringe 5 ml	Yingmed
9	EDTA tube	Plastilab ,China
10	Electric oven	Memmert, Germany
11	Electric shaker	Bioneer ,England
12	Eppendorf tube 1.5 ml	China Mheco ,China
13	Graduated cylinder	Volac, England
14	Medical Cotton	Vitafour
15	Micropipette (10_100 μ L)	HUMAN Humapette, Germany
16	Micropipette (100_1000 μ L)	HUMAN Humapette, Germany
17	Sample vial 2ml	Newstar
18	Screwed glass test tube	China Mheco ,China
19	Separating funnel	Simax, Czech Republic
20	Stand	China Mheco ,China
21	Syringe Filter	Newstar
22	Test tube	China Mheco ,China
23	Test tube rack	China Mheco ,China
24	Thin layer chromatography plate (TLC)	Chmlab, Spanish
25	Uv source	Cleaver ,England
26	Volumetric flask	Volac, England

Kits and chemicals**Table (2-2): Kits and chemicals used in the study.**

NO	Chemicals & Apparatus	Manufactures
1	Acetone	HiMedia -India
2	Benzene	HiMedia -India
3	Chloroform	HiMedia -India
4	Human SERPINA4(Kallistatin)	Elabscience® - USA
5	Methanol	HiMedia -India
6	Proteinase k kit	Intron Biotechnology-Korea
7	Water HPLC	Biosolve Chimie SARL- France

2.3 Methodology**2.3.1. Body Mass Index Measurement**

Obesity was categorized using the body mass index (BMI) according to the traditional World Health Organization (WHO) which was calculated from the following equation: BMI (Kg/m²) = Weight (kg) / Height (m²).

The ranges of (BMI) were categorized into groups **Namjou *et al*(2021)**, see table (2-3).

Table (2-3): Body Mass Index. Weight status

	BMI(Kg/m ²)
Underweight	≤ 18.5
Normal weight	18.5-24.9
Overweight	25-29.9
Obese	≥ 30

2.3.2. Waist Hip Ratio Measurement

The Waist Hip Ratio (WHR) ≥ 0.85 is the diagnostic standard for abdominal obesity in women according to WHO (**Lee *et al.*, 2022**).

The formula is: WHR= waist (cm) / hip (cm)

2.3.3 Anthropometric Measurements

Table (2-4): Formulas of Anthropometric Measurements	References
Anthropometric measurements	
BAI % = hip circumference (cm)/height (m) ^{1.5} – 18	(Li <i>et al.</i> , 2016)
VAI (Female) = [WC (cm)/ (36.58 + (1.89 × BMI))] × [Triglycerides (mmol/l)/0.81] × [1.52/HDL (mmol/l)]	(Amato <i>et al.</i> , 2014)
LAP (Female) = (WC in cm – 58) × Triglycerides in mmol/L	(Ahn <i>et al.</i> , 2019)

BAI: body adiposity index; , LAP: lipid accumulation product and VAI: visceral adiposity index

2.3.4. Basal Metabolic Rate

The Mifflin-St Jeor for Basal Metabolic Rate (BMR) has used the following equation: BMR (Female): [(10 × weight in kg) + (6.25 × height in cm) - (5 × age in years) – 161] (Mifflin *et al.*, 1990)

2.4.1 Qualitative analysis of serum Zearalenone

Following the steps outlined by (Al-Musoui, 2015), all glassware used in the laboratory work was sterilized daily in an autoclave at 120°C for 15 minutes to maintain clean and sterile conditions. To reduce the risk of contamination, work surfaces were also wiped thoroughly with 70% isopropyl alcohol before starting each day's procedures.

2.4.2 Standard ZEN Preparation for TLC

A standard solution of Zearalenone (ZEN) was prepared by accurately dissolving the powdered form of ZEN in acetonitrile to obtain a final concentration of 10 ng/L. Acetonitrile was chosen as the solvent due to its high compatibility with ZEN, allowing for efficient dissolution. The solution was vortexed gently to ensure complete solubilization. After preparation, the standard solution was stored in a tightly sealed amber glass vial at 4°C to protect it from light and maintain its chemical stability. This standard was later used as a reference in thin-layer

chromatography (TLC) analysis to identify the presence of ZEN in serum samples by comparing retention factors and fluorescence under UV light.

2.4.3 Proteinase K Solution

A working solution of Proteinase K was prepared by dissolving 22 mg of Proteinase K powder (Molecular weight: 28,900 Da) in 1.1 mL of sterile distilled water. The mixture was gently vortexed to ensure complete dissolution. The prepared solution was stored at 4°C and used in the extraction process as a proteolytic agent to aid in the digestion of proteins and facilitate the release of target compounds from the serum matrix.

2.4.4. Extraction of Zearalenone (ZEN) from Serum Samples

- Serum samples (500 µL) were transferred into sterile test tubes.
- To each sample, 50 µL of Proteinase K solution was added, and the mixture was incubated at room temperature for 10 minutes to allow enzymatic digestion.
- The samples were then centrifuged at $5000 \times g$ for 15 minutes.
- After centrifugation, the clear supernatant was carefully collected, and the precipitate was discarded.
- One milliliter of chloroform (twice the volume of serum) was added to each supernatant.
- The mixture was vigorously shaken using an electric shaker to promote thorough mixing and phase separation.
- Two distinct layers formed: an organic chloroform layer and an aqueous serum layer.
- The chloroform layer, which contained the extracted ZEN, was separated using a separating funnel and transferred into sterile glass tubes.
- Finally, the chloroform extracts were left to evaporate at room temperature before proceeding to further qualitative analysis.

2.4.5. Detection of Zearalenone (ZEN) by Thin Layer Chromatography (TLC)

Silica gel-coated TLC plates measuring 20×10 cm were used for the analysis. Before use, the plates were activated by heating them in a benzene-saturated chamber at 80°C for 20 minutes

to prepare the silica surface for better separation. The mobile phase was prepared by mixing 90 mL of benzene with 10 mL of acetone.

Using a fine capillary tube, 50 μL of each standard and sample solution was carefully applied onto the plate, 1.5 cm from the bottom edge. The plates were then placed vertically into a developing chamber containing the mobile phase, allowing the solvent to rise until it nearly reached the top of the plate.

After development, the plates were removed and left to air dry at room temperature. Visualization was performed under UV light at 360 nm, where Zearalenone was identified by

comparing the fluorescence and retention factor (Rf) of the sample spots to those of the standard.

2.4.6. Analysis of Zearalenone (ZEN) by High Performance Liquid Chromatography (HPLC)

1. Preparation of Mobile Phase:

- The mobile phase was prepared by mixing 240 mL of methanol with 60 mL of HPLC-grade water.
- A dilution solvent for sample preparation was made by mixing 80 mL of methanol with 20 mL of HPLC-grade water (80:20, v/v).

2. Sample Preparation:

- A volume of 400 μ L of each serum sample was placed into a clean vial and diluted to a final volume of 1 mL.
- The mixture was vortexed thoroughly to ensure complete mixing.
- Samples were centrifuged at an appropriate speed for approximately 15 minutes to remove precipitates and impurities.
- The clear supernatant was then withdrawn for injection.

3. HPLC Analysis:

- Separation was carried out using a Shimadzu liquid chromatography system (Shimadzu, Japan, Kyoto), model 10AV-LC, equipped with a binary delivery pump (model LC-10A).
- Chromatographic separation was achieved using an HCMA-BIO 1000 C18 column (3 μ m particle size, 50 $\times$ 4.6 mm I.D) maintained at ambient temperature.
- The mobile phase was delivered at a flow rate of 0.7 mL/min.
- An injection volume of 50 μ L was introduced into the system via an autosampler.
- The eluted peaks were monitored using an Fx 10A fluorescence detector at two different excitation wavelengths to ensure sensitive detection of Zearalenone.
- All system parameters were set according to the standard operating procedure before injection.

2.5. Elabscience® Human SERPINA4(Kallistatin) ELISA Kit**Catalog No: E-EL-H5550****Product size: 96T/48T/24T/96T*5****Intended use**

The ELISA kit was intended for the in vitro quantitative determination of Human SERPINA4 concentrations in serum, plasma, and other biological fluids.

Table (2-5) Specifications and performance of the Human SERPINA4 ELISA kit.

Item	
Sensitivity	9.38 ng/ml
Detection Range	15.63-1000 ng/ml
Specificity	Human SERPINA4 was recognized by this kit in samples. No significant cross-reactivity or interference between Human SERPINA4 and analogues was observed.
Repeatability	Coefficient of variation is < 10%

Test Principle:

The Sandwich-ELISA principle was used in this ELISA kit. The micro ELISA plate provided in the kit had been pre-coated with an antibody specific to Human SERPINA4. Samples or standards were added to the micro ELISA plate wells and were combined with the specific antibody. Then, a biotinylated detection antibody specific for Human SERPINA4 and an Avidin-Horseradish Peroxidase (HRP) conjugate were added successively to each microplate well and were incubated. Free components were washed away. The substrate solution was added to each well. Only those wells that contained Human SERPINA4, biotinylated detection antibody, and Avidin-HRP conjugate appeared blue in color. The enzyme-substrate reaction was terminated by the addition of stop solution, and the color was changed to yellow. The optical density (OD) was measured spectrophotometrically at a wavelength of 450 ± 2 nm. The OD value was considered proportional to the concentration of Human SERPINA4. The

concentration of Human SERPINA4 in the samples was calculated by comparing the OD of the samples to the standard curve.

Kit Components & Storage:

An unopened kit was stored at 2–8 °C for 1 month. If the kit was not intended to be used within 1 month, the items were stored separately according to the following conditions once the kit was received.

Table (2-6) Components and recommended storage conditions of the Human SERPINA4 ELISA kit.

Item	Specifications	Storage
Micro ELISA Plate	96T: 8 wells ×12 strips 48T: 8 wells ×6 strips 24T: 8 wells ×3 strips 96T*5: 5 plates, 96T	-20°C, 12 months
Reference Standard	96T: 2 vials 48T/24T: 1 vial 96T 5: 10 vials	-20°C, 12 months
Concentrated Biotinylated Detection Ab (100×)	96T: 1 vial, 120 µL 48T/24T: 1 vial, 60 µL 96T 5: 5 vials, 120 µL	
Concentrated HRP Conjugate (100x)	96T: 1 vial, 120 µL 48T/24T: 1 vial, 60 µL 96T 5: 5 vials, 120 µL	-20°C(Protect from light), 12 months
Reference Standard & Sample Diluent	96T/48T/24T: 1 vial, 20 mL 96T*5: 5 vials, 20 mL	2-8°C, 12 months
Biotinylated Detection Ab Diluent	96T/48T/24T: 1 vial, 14 mL 96T5: 5 vials, 14 mL	2-8°C, 12 months
HRP Conjugate Diluent	96T/48T/24T: 1 vial, 14 mL 96T 5: 5 vials, 14 mL	
Concentrated Wash Buffer(25x)	96T/48T/24T: 1 vial, 30 mL 96T 5: 5 vials, 30 mL	
Substrate Reagent	96T/48T/24T: 1 vial, 10 mL 96T 5: 5 vials, 10 mL	2-8°C(Protect from light)
Stop Solution	96T/48T/24T: 1 vial, 10 mL 96T 5: 5 vials, 10 mL	2-8°C
Plate Sealer	96T/48T/24T: 5 pieces 96T*5: 25 pieces	
Product Description	1 copy	
Certificate of Analysis	1 copy	

Reagent Preparation:

1. All reagents were brought to room temperature (18–25 °C) before use. If the kit was not used up in one assay, only the necessary strips and reagents for the present experiment were taken out, and the remaining strips and reagents were stored under the required conditions.

2. Wash Buffer: 30 mL of Concentrated Wash Buffer was diluted with 720 mL of deionized or distilled water to prepare 750 mL of Wash Buffer. Note: If crystals had formed in the concentrate, it was warmed in a 40 °C water bath and was mixed gently until the crystals were completely dissolved.

3. Standard working solution: The standard was centrifuged at 10,000×g for 1 minute. Then, 1 mL of Reference Standard & Sample Diluent was added, and the tube was left to stand for 10 minutes and was gently inverted several times. After it had fully dissolved, it was mixed thoroughly with a pipette. This reconstitution produced a working solution of 1000 ng/mL. (Alternatively, 1 mL of Reference Standard & Sample Diluent was added, the tube was left to stand for 1–2 minutes, and then was thoroughly mixed using a vortex meter at low speed. Bubbles generated during vortexing were removed by centrifugation at a relatively low speed). Serial dilutions were then made as needed. The recommended dilution gradient was as follows: 1000, 500, 250, 125, 62.5, 31.25, 15.63, and 0 ng/mL.

Dilution method: Seven EP tubes were prepared, and 500 µL of Reference Standard & Sample Diluent was added to each tube. Then, 500 µL of the 1000 ng/mL working solution was pipetted into the first tube and was mixed to produce a 500 ng/mL working solution. Subsequently, 500 µL of the solution from the former tube was transferred into the next one, and the process was repeated accordingly. Note: The last tube was regarded as a blank and no solution was

pipetted into it from the previous tube. The gradient diluted standard working solutions were prepared just before use.

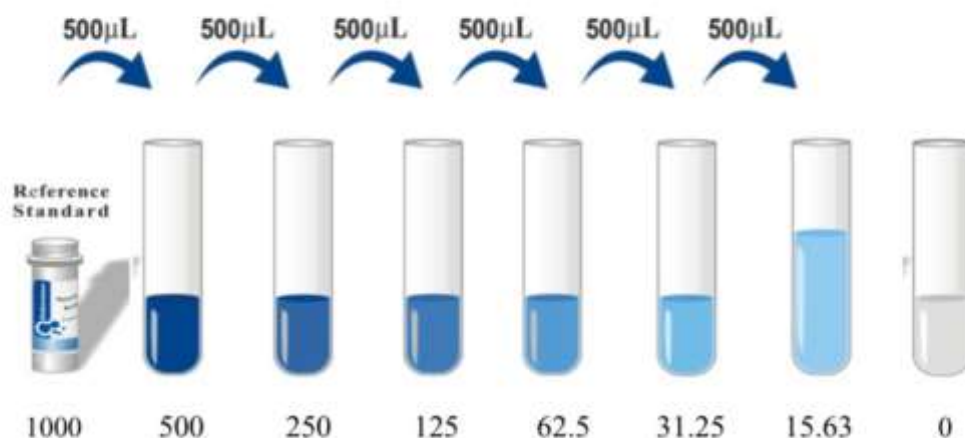


Figure (2-2). Preparation of the serial dilutions for the Human SERPINA4 standard curve (1000–0 ng/mL) as illustrated in the ELISA kit manual.

Image source: Elabscience Human SERPINA4 ELISA Kit Manual, Catalog No. [E-EL-H5550].

4. Biotinylated Detection Antibody Working Solution:

The required amount was calculated before the experiment (100 µL/well). Slightly more than the calculated volume was prepared. The Concentrated Biotinylated Detection Antibody was centrifuged at 800×g for 1 minute. Then, the 100× Concentrated Biotinylated Detection Antibody was diluted to a 1× working solution using Biotinylated Detection Antibody Diluent (at a ratio of 1:99). The working solution was prepared just before use.

5. HRP Conjugate Working Solution:

HRP Conjugate (HRP-conjugated avidin) was used. The required amount was calculated prior to the experiment (100 µL/well), and slightly more than the calculated volume was prepared. The Concentrated HRP Conjugate was centrifuged at 800×g for 1 minute. Then, the 100×

Concentrated HRP Conjugate was diluted to a 1× working solution using HRP Conjugate Diluent (at a ratio of 1:99). The working solution was prepared just before use.

Assay Procedure:

1. Wells for diluted standards, blanks, and samples were determined. Then, 100 µL of each standard dilution, blank, and sample was added into the appropriate wells. (It was recommended that all samples and standards be assayed in duplicate. The dilution ratio of samples was determined through preliminary experiments or based on technical support recommendations). The plate was covered with the sealer provided in the kit and was incubated for 90 minutes at 37 °C. Note: Solutions were added to the bottom of the micro ELISA plate wells, though touching the inner wall and causing foaming was avoided as much as possible.
2. The liquid from each well was decanted without washing. Immediately, 100 µL of Biotinylated Detection Antibody working solution was added to each well. The plate was covered with a new sealer and was incubated for 1 hour at 37 °C.
3. The solution from each well was decanted, and 350 µL of wash buffer was added to each well. The wells were soaked for 1 minute, then the solution was aspirated or decanted, and the wells were patted dry against clean absorbent paper. This wash step was repeated three times. Note: A microplate washer could have been used in this step and in other wash steps. The tested strips were used immediately after the wash step. Wells were not allowed to dry.
4. 100 µL of HRP Conjugate working solution was added to each well. The plate was covered with a new sealer and was incubated for 30 minutes at 37 °C.
5. The solution from each well was decanted, and the wash process was repeated five times as described in step 3.
6. 90 µL of Substrate Reagent was added to each well. The plate was covered with a new sealer and was incubated for approximately 15 minutes at 37 °C, protected from light. Note: The reaction time could have been shortened or extended according to the actual color change, but

was not allowed to exceed 30 minutes. The Microplate Reader was preheated for about 15 minutes before OD measurement.

7. 50 μ L of Stop Solution was added to each well. Note: The stop solution was added in the same order as the substrate solution.

8. The optical density (OD value) of each well was immediately determined using a microplate reader set to 450 nm.

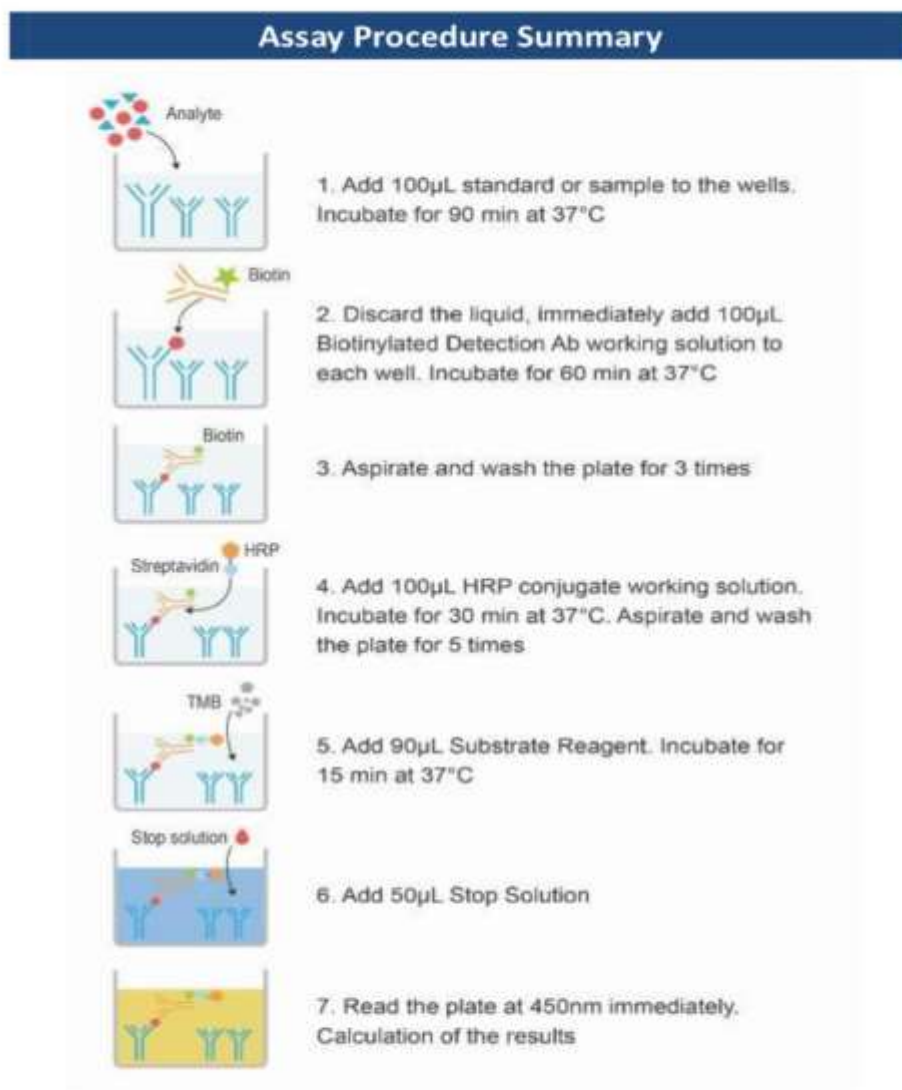


Figure (2-3) Illustrated summary of the ELISA procedure steps used in the Human SERPINA4 assay.

Image source: Elabscience Human SERPINA4 ELISA Kit Manual, Catalog No. [E-EL-H5550], China.

Calculation of Results:

The duplicate readings for each standard and sample were averaged, then the average optical density of the zero standard was subtracted from each value. A four-parameter logistic curve was plotted on log-log graph paper, with the standard concentration on the x-axis and the OD values on the y-axis. If the OD of a sample had surpassed the upper limit of the standard curve, the sample was re-tested with an appropriate dilution. The actual concentration was obtained by multiplying the calculated concentration by the dilution factor.

Statistical Analysis:

Information from the questionnaire from all participants was entered into a data sheet and was assigned a serial identifier number. Multiple entries were used to avoid errors. The data analysis for this work was generated using The Statistical Package for the Social Sciences software, version 28.0 (IBM, SPSS, Chicago, Illinois, USA,) and the Real Statistics Resource Pack software for Mac (Release 7.2) of the resource pack for Excel 2016. Copyright (2013 – 2020). Descriptive statistics were performed on the participants' data of each group. Values were illustrated by n (%) for categorical. The distribution of the data was checked using the Shapiro-Wilk test as numerical means of assessing normality.

The association between the analyzed factors was estimated using odds ratios (ORs) and a 95% Confidence Interval Range which was calculated by a non-conditional logistic regression.

Significant differences in categorical variables among the parameters were confirmed through analytical statistical tests. Results of all hypothesis tests with p-values <0.05 (two-side) were considered to be statistically significant.

The optimal threshold with high specificity and sensitivity for PCOS phenotypes was detected using receiver operating characteristic (ROC) analysis.

CHAPTER THREE

Results



Results:

Table (3-1) presents the sociodemographic characteristics and phenotypic variations among women diagnosed with Polycystic Ovary Syndrome (PCOS). The cohort was categorized into four phenotypes: Classic PCOS (Anovulation, Hyperandrogenism, Polycystic Ovaries - A, HA, PCO), Classic PCOS (A, HA), Ovulatory PCOS (HA, PCO), and Normo-androgenic PCOS (A, PCO).

The mean age of participants ranged from 23.55 years in the Classic PCOS (A, HA) group to 26.58 years in the Normo-androgenic PCOS (A, PCO) group. Body Mass Index (BMI) also varied across the phenotypes, with the Classic PCOS (A, HA, PCO) group exhibiting the highest mean BMI at 29.76 Kg/m², though the Classic PCOS (A, HA) group had the lowest at 26.25 Kg/m². Waist-to-hip ratio (WHR) showed less variation, ranging from 0.79 in the Classic PCOS (A, HA) group to 0.83 in the Classic PCOS (A, HA, PCO) group.

Regarding family history of PCOS, the Ovulatory PCOS (HA, PCO) group reported the highest proportion of no family history (60%), though the Classic PCOS (A, HA, PCO) group had the highest percentage with a positive family history (57%). The duration of the disease also differed, with the Classic PCOS (A, HA, PCO) group having the longest mean duration at 5.40 years, and the Normo-androgenic PCOS (A, PCO) group the shortest at 3.58 years.

Table (3-1). Sociodemographic characteristic and phenotypes of PCOS

		<i>Classic PCOS (A, HA, PCO)</i>	<i>Classic PCOS (A, HA)</i>	<i>Ovulatory PCOS (HA, PCO)</i>	<i>Normo-androgenic PCOS (A, PCO)</i>
<i>Age Years</i>		25.85	23.55	26.20	26.58
<i>BMI Kg/m²</i>		29.76	26.25	28.49	27.45
<i>WHR</i>		0.83	0.79	0.82	0.80
<i>Family</i>	<i>No</i>	43%	55%	60%	47%
<i>History</i>	<i>Yes</i>	57%	45%	40%	53%
<i>Duration of disease (Years)</i>		5.40	3.73	4.67	3.58

* Body mass index (BMI), waist-to-hip ratio (WHR)

Table (3-2) showed the prevalence of various clinical manifestations and their association with the four identified PCOS phenotypes: Classic PCOS (A, HA, PCO), Classic PCOS (A, HA), Ovulatory PCOS (HA, PCO), and Normo-androgenic PCOS (A, PCO).

Hirsutism was most prevalent in the Classic PCOS (A, HA, PCO) group, affecting 85% of individuals, followed by Classic PCOS (A, HA) at 64%. In contrast, Ovulatory PCOS (HA, PCO) and Normo-androgenic PCOS (A, PCO) showed lower rates of hirsutism at 47% and 53%, respectively. Acne was reported in 53% of the Classic PCOS (A, HA, PCO) group, 36% of the Classic PCOS (A, HA) group, 40% of the Ovulatory PCOS (HA, PCO) group, and 37% of the Normo-androgenic PCOS (A, PCO) group. Alopecia was most common in the Ovulatory PCOS (HA, PCO) group (53%) and Classic PCOS (A, HA) group (54%), but less so in the Normo-androgenic PCOS (A, PCO) group (32%).

Regarding types of infertility, Type I infertility was equally prevalent (50%) in both Classic PCOS (A, HA) and Ovulatory PCOS (HA, PCO) groups, though it was less common in Normo-androgenic PCOS (A, PCO) at 23%. Conversely, Type II infertility was notably high in the Normo-androgenic PCOS (A, PCO) group (77%) and the Classic PCOS (A, HA, PCO) group (67%). The rate of reported abortions ranged from 15% in Classic PCOS (A, HA, PCO) to 27% in Classic PCOS (A, HA).

Menstrual cycle regularity showed significant differences across phenotypes. An overwhelming majority of individuals in Classic PCOS (A, HA, PCO) (94%), Classic PCOS (A, HA) (100%), and Normo-androgenic PCOS (A, PCO) (95%) reported irregular menstrual cycles. In stark contrast, 73% of women with Ovulatory PCOS (HA, PCO) reported regular menstrual cycles, defining them as typically within 21-35 days over a 12-month period, though irregular cycles consistently fell outside this range.

Table (3-2). Clinical variables and association with phenotypes of PCOS

		<i>Classic PCOS (A, HA, PCO)</i>	<i>Classic PCOS (A, HA)</i>	<i>Ovulatory PCOS (HA, PCO)</i>	<i>Normo- androgenic PCOS (A, PCO)</i>
<i>Hirsutism</i>	<i>Yes</i>	85%	64%	47%	53%
	<i>No</i>	15%	36%	53%	47%
<i>Acne</i>	<i>Yes</i>	53%	36%	40%	37%
	<i>No</i>	47%	64%	60%	63%
<i>Alopecia</i>	<i>Yes</i>	40%	54%	53%	32%
	<i>No</i>	60%	46%	47%	68%
<i>Type of infertility</i>	<i>Type I</i>	37%	50%	50%	23%
	<i>Type II</i>	67%	50%	50%	77%
<i>Abortion</i>	<i>Yes</i>	15%	27%	20%	16%
	<i>No</i>	85%	73%	80%	84%
<i>Regularity of mc.</i>	<i>regular</i>	6%	0%	73%	5%
	<i>irregular</i>	94%	100%	27%	95%

* Hirsutism, androgenetic alopecia, acne (dichotomously as presence or absence);

*Regular mc. typically within 2-20 days over 12 months. Irregular, if the length consistently falls outside the 21-35 day range,

Table (3-3) presents the median (range) values for key reproductive hormones and sex hormone-binding globulin (SHBG) in PCOS patients, stratified by infertility type (Type 1, Type 2) and in an "Un-married" group.

Luteinizing Hormone (LH) levels were relatively consistent across the groups, with medians of 7.8 mIU/ml for Infertility Type 1, 8.4 mIU/ml for Infertility Type 2, and 8.8 mIU/ml for the Un-married group. Follicle-Stimulating Hormone (FSH) also showed similar median values: 5.5 mIU/ml for Infertility Type 1, and 6.3 mIU/ml for both Infertility Type 2 and Un-married individuals.

Prolactin (PRO) levels had a median of 17 ng/ml for Infertility Type 1, 20 ng/ml for Infertility Type 2, and 16 ng/ml for the Un-married group. Testosterone (TEST) levels, a key indicator in PCOS, showed variations. The Infertility Type 1 group had the highest median testosterone at 69 ng/dl, followed by the Un-married group at 52 ng/dl, and the Infertility Type 2 group with the lowest median at 47 ng/dl.

Sex Hormone Binding Globulin (SHBG) levels exhibited an inverse relationship with testosterone, being highest in the Infertility Type 1 group (39 nmol/L) and progressively lower in the Infertility Type 2 (28 nmol/L) and Un-married (24 nmol/L) groups. Finally, Estradiol (E2) levels were highest in the Un-married group (64 pg/ml), followed by the Infertility Type 1 group (54 pg/ml), and lowest in the Infertility Type 2 group (41 pg/ml).

Table (3-3). Reproductive variables (Median (mini-max)) among PCOS patients groups based on types of infertility

	<i>Infertility Type 1</i>	<i>Infertility Type 2</i>	<i>Un-married</i>
<i>LH (mIU/ml)</i>	7.8 (3.0-20)	8.4 (0.33-21)	8.8 (1.6-28)
<i>FSH (mIU/ml)</i>	5.5 (1.2-9.9)	6.3 (3.2-15)	6.3 (2.8-9.4)
<i>PRO (ng/ml)</i>	17 (5.2-48)	20 (6.0-76)	16 (6.9-93)
<i>TEST (ng/dl)</i>	69 (6.0-143)	47 (8.0-98)	52 (5-140)
<i>SHBG (nmol/L)</i>	39 (26-43)	28 (11-47)	24 (17-38)
<i>E2 (pg/ml)</i>	54 (33-180)	41 (29-132)	64 (30-185)

* LH (Luteinizing hormone), FSH (Follicle-stimulating hormone), SHBG: Sex hormone Binding Globulin, E2: Estradiol

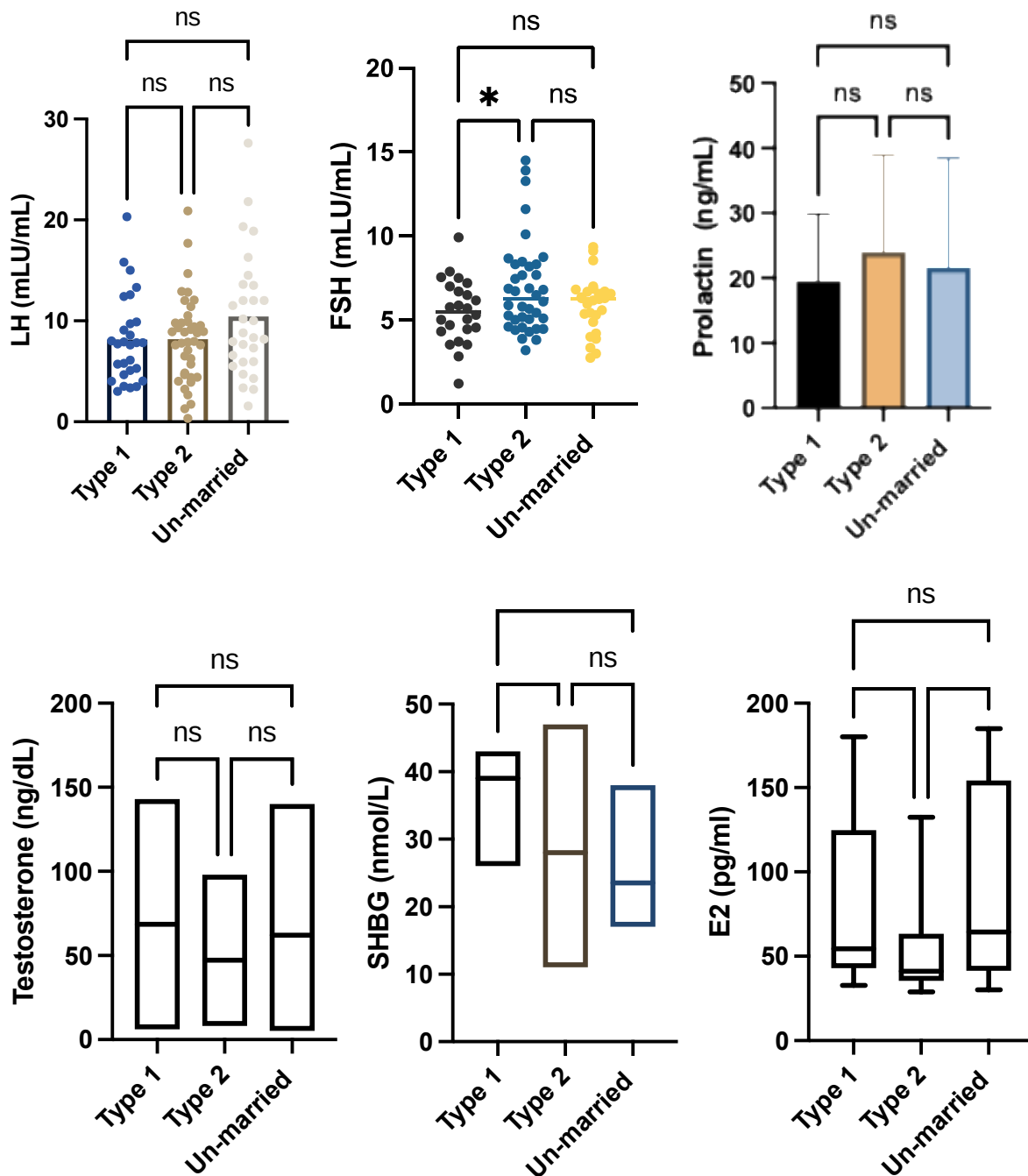


Figure (3-1). The distribution of serum Reproductive variables among PCOS patients groups based on types of infertility (Post Hoc ANOVA test was *: significant at $p \leq 0.05$, **: significant at $p \leq 0.01$, ***: significant at $p \leq 0.001$, ****: significant at $p \leq 0.0001$)

Table (3-4) presents the median (and range) values for several metabolic indices, including body adiposity index (BAI), visceral adiposity index (VAI), lipid accumulation product (LAP), and basal metabolic rate (BMR), among PCOS patients categorized by infertility type (Type 1, Type 2) and an "Un-married" group.

The Body Adiposity Index (BAI) showed nearly similar median values across all groups: 30 for Infertility Type 1, 33 for Infertility Type 2, and 32 for the Un-married group, indicating comparable levels of body fat distribution relative to height.

Visceral Adiposity Index (VAI) displayed some variation, with the Infertility Type 2 group having the highest median VAI at 3.4, suggesting a greater accumulation of visceral fat in this subgroup. The Infertility Type 1 group had a median VAI of 2.4, though the Un-married group's median was 2.9.

The Lipid Accumulation Product (LAP) also varied, with the Infertility Type 2 group exhibiting the highest median LAP at 94. The Infertility Type 1 group had a median LAP of 67, and the Un-married group's median was 76. These values suggest that the Infertility Type 2 group might have a higher risk of cardiovascular metabolic alterations.

Basal Metabolic Rate (BMR) demonstrated relatively consistent median values across the groups: 1390 for Infertility Type 1, 1478 for Infertility Type 2, and 1422 for the Un-married group. The range of BMR values indicates individual variability within each group.

Table (3-4). Biochemical evaluation of metabolic indices (Median (mini-max)) among PCOS groups based on types of infertility.

	<i>Infertility Type 1</i>	<i>Infertility Type 2</i>	<i>Un-married</i>
<i>BAI</i>	30 (25-53)	33 (19-70)	32 (19-53)
<i>VAI</i>	2.4 (1.9-4)	3.4 (2.3-5.4)	2.9 (1.7-4.5)
<i>LAP</i>	67 (37-348)	94 (10-583)	76 (9.1-406)
<i>BMR</i>	1390 (1192-1898)	1478 (1145-1894)	1422 (1232-1904)

*body adiposity index (BAI), visceral adiposity index (VAI), lipid accumulation product (LAP), and basal metabolic rate (BMR)

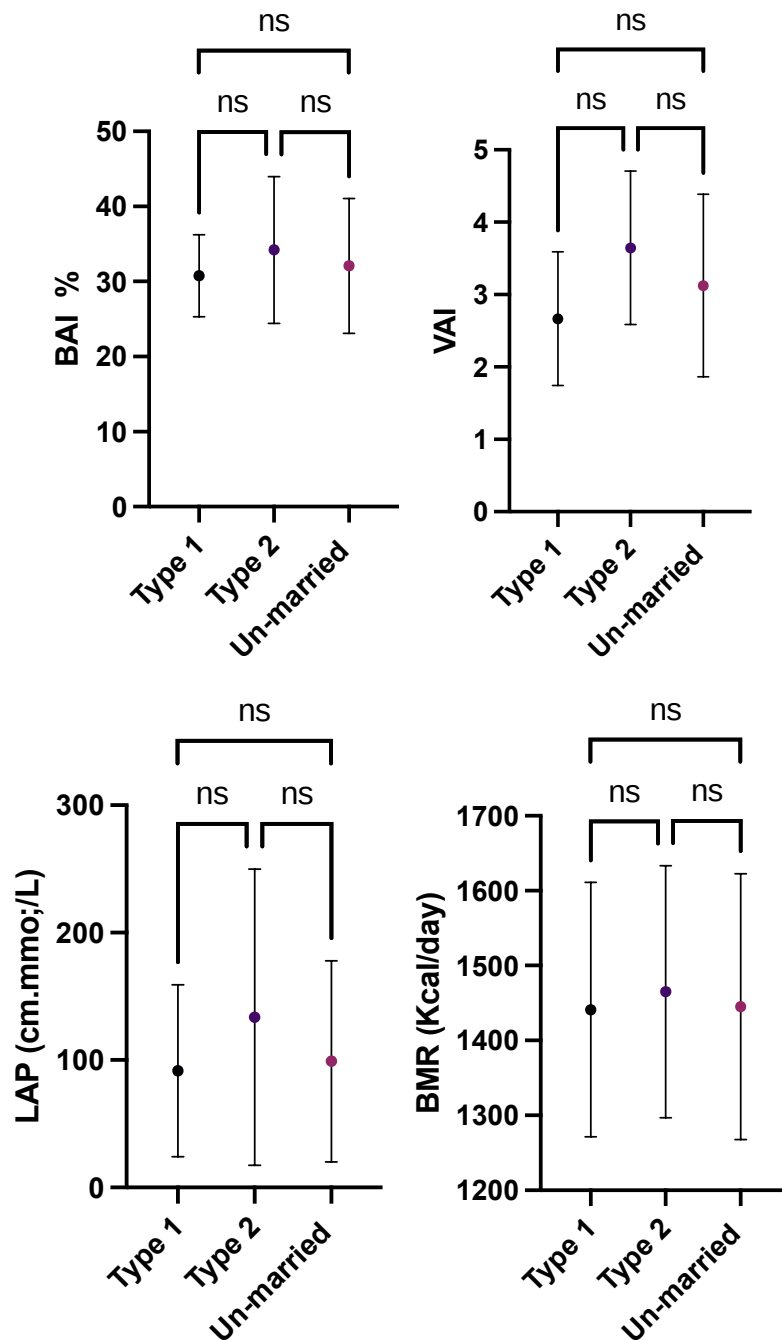


Figure (3-2). The distribution of evaluation metabolic incised among PCOS patients groups based on types of infertility (Post Hoc ANOVA test was *: significant at $p \leq 0.05$, **: significant at $p \leq 0.01$, ***: significant at $p \leq 0.001$, ****: significant at $p \leq 0.0001$)

Table (3-5) presents the median (and range) levels of Kallistatin and Zearalenone across different groups of PCOS patients (Infertility Type 1, Infertility Type 2, Un-married) and a Healthy Control group.

The median Kallistatin levels varied considerably among the groups. The Healthy Control group exhibited the highest median Kallistatin level at 66 (ranging from 16 to 342). Among the PCOS groups, Infertility Type 1 showed a median of 42 (range: 4.2-271), and Infertility Type 2 had a median of 37 (range: 1.4-247). Notably, the Un-married PCOS group displayed the lowest median Kallistatin level at 9.6 (range: 2.0-68).

For Zearalenone, the median levels were relatively consistent across the Infertility Type 1 (4.8 ng/L, range: 2.7-11) and Infertility Type 2 (4.8 ng/L, range: 2.1-12) groups. The Healthy Control group had a median Zearalenone level of 4.2 ng/L (range: 2.1-8.2), which was the lowest. The Un-married PCOS group showed the highest median Zearalenone level at 7.2 ng/L (range: 3.3-16).

The Healthy Control group consistently showed higher Kallistatin levels compared to all PCOS patient groups. This aligns with recent research suggesting that reduced kallistatin levels may be associated with PCOS and could serve as a biomarker, particularly linked to hyperandrogenism and inflammation. The Un-married PCOS group, in particular, exhibited a significantly lower median Kallistatin level compared to the other PCOS subgroups and healthy controls, which might suggest a more severe inflammatory or metabolic profile in this specific cohort, or perhaps indicate different underlying mechanisms in women who remain unmarried and also suffer from PCOS.

Zearalenone levels were broadly similar across the infertility types in PCOS patients and the healthy controls, with a slight elevation observed in the Un-married PCOS group. Zearalenone is a mycoestrogen known to disrupt endocrine function and impact fertility, acting similarly to estrogen. The observed levels are within the range that might be considered concerning given Zearalenone's known endocrine-disrupting properties and potential links to reproductive dysfunction.

Table (3-5). Mean level of Kallistatin and Zearalenone (Median (mini-max)) among PCOS patients groups based on types of infertility

	<i>Infertility Type 1</i>	<i>Infertility Type 2</i>	<i>Un-married</i>	<i>Healthy Control</i>
Kallistatin	42 (4.2-271)	37 (1.4-247)	9.6 (2.0-68)	66 (16-342)
Zearalenone	4.8 (2.7-11)	4.8 (2.1-12)	7.2 (3.3-16)	4.2 (2.1-8.2)

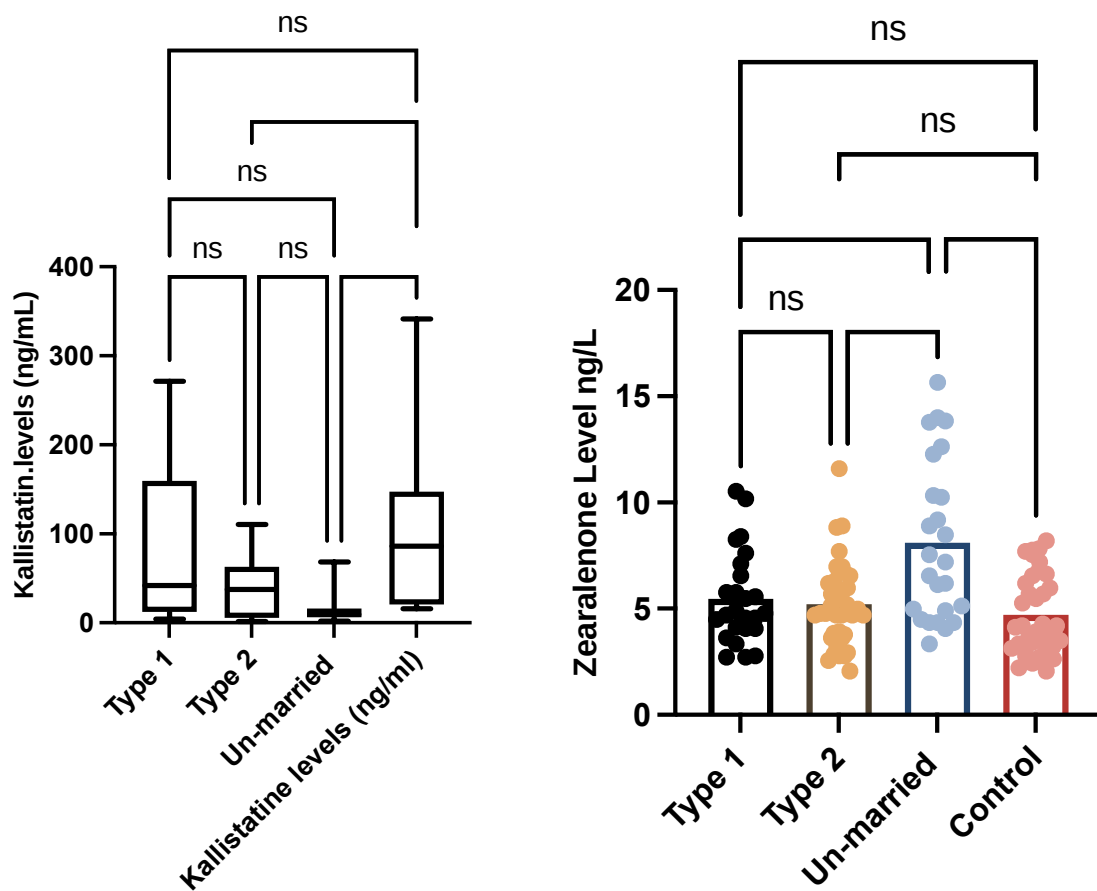


Figure (3-3). The distribution of serum level of Kallistatin and Zearalenone among PCOS patients groups based on types of infertility (Post Hoc ANOVA test was *: significant at $p \leq 0.05$, **: significant at $p \leq 0.01$, ***: significant at $p \leq 0.001$, ****: significant at $p \leq 0.0001$)

Table (3-6) illustrates the median (and range) of reproductive hormone levels in PCOS patients, categorized by whether they experience irregular or regular menstrual cycles (mc).

For Luteinizing Hormone (LH), patients with irregular menstrual cycles had a median of 8.3 mIU/ml (range: 1.3-28), though those with regular cycles showed a slightly lower median of 7.8 mIU/ml (range: 3.4-20). In contrast, Follicle-Stimulating Hormone (FSH) levels were notably higher in patients with regular menstrual cycles, with a median of 7.7 mIU/ml (range: 3.0-19), compared to 5.8 mIU/ml (range: 1.2-9.9) in those with irregular cycles.

Prolactin levels were relatively similar between the groups, with a median of 17 ng/ml (range: 5.2-93) for irregular cycles and 14 ng/ml (range: 7.5-32) for regular cycles. Testosterone levels, a key indicator of hyperandrogenism in PCOS, were distinctly higher in the irregular mc. group, with a median of 61 ng/dl (range: 5.0-143), as opposed to 45 ng/dl (range: 9.0-97) in the regular mc. group.

Sex Hormone Binding Globulin (SHBG) showed an inverse pattern to testosterone; patients with regular menstrual cycles had a higher median SHBG of 43 nmol/L (range: 34-47), whereas those with irregular cycles had a lower median of 29 nmol/L (range: 13-47). Finally, Estradiol (E2) levels were higher in the regular mc. group (median 68 pg/ml, range: 33-166) compared to the irregular mc. group (median 45 pg/ml, range: 29-173).

There was a clear difference in hormonal profiles based on menstrual cycle regularity in PCOS patients. Individuals with irregular menstrual cycles, a hallmark of PCOS, typically present with higher median Testosterone levels and lower SHBG levels compared to those with regular cycles. This hormonal imbalance (elevated androgens and reduced SHBG, which binds free androgens) is consistent with the anovulatory or oligo-ovulatory nature of irregular cycles in PCOS.

Conversely, patients with regular menstrual cycles, despite having PCOS, exhibit higher median FSH and E2 levels, and lower Testosterone and higher SHBG. The higher FSH in the regular cycle group likely indicates better follicular development and function. These distinctions underscore the heterogeneity of PCOS phenotypes and suggest that menstrual regularity is strongly associated with different underlying hormonal characteristics.

Table (3-6). Reproductive variables (Median (mini-max)) among PCOS patients groups based on regularity of mc.

	<i>Irregular mc.</i>	<i>Regular mc.</i>
<i>LH (mIU/ml)</i>	8.3 (1.3-28	7.8 (3.4-20
<i>FSH (mIU/ml)</i>	5.8 (1.2-9.9	7.7 (3.0-19
<i>Prolactin. (ng/ml)</i>	17 (5.2-93	14 (7.5-32
<i>Testosterone (ng/dl)</i>	61 (5.0-143	45 (9.0-97
<i>SHBG (nmol/L)</i>	29 (13-47	43 (34-47
<i>E2 (pg/ml)</i>	45 (29-173	68 (33-166

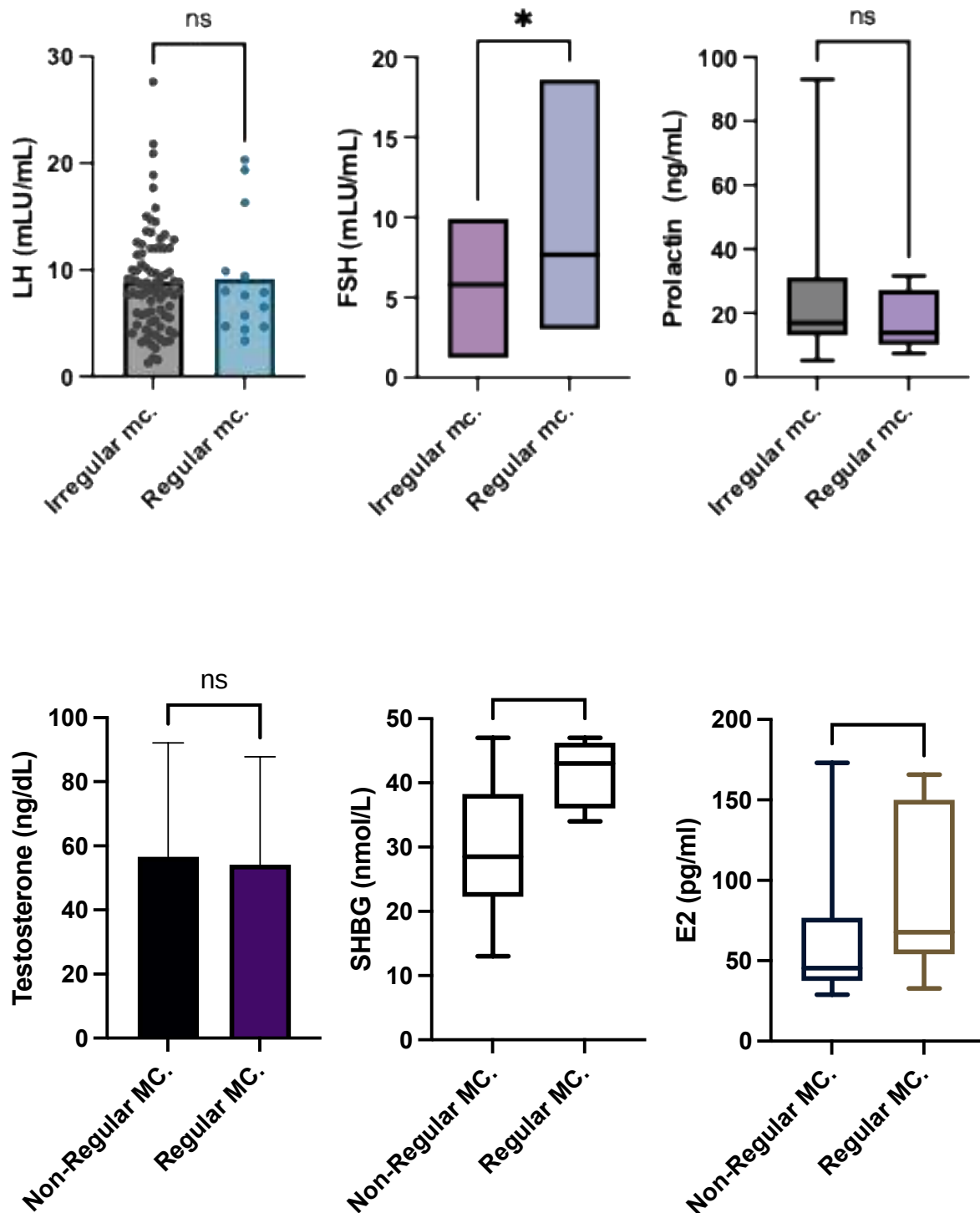


Figure (3-4). The distribution of serum Reproductive variables among PCOS patients groups based on regularity of mc. (Post Hoc ANOVA test was *: significant at $p \leq 0.05$, **: significant at $p \leq 0.01$, ***: significant at $p \leq 0.001$, ****: significant at $p \leq 0.0001$)

Table (3-7) presents the median (and range) values for metabolic indices, including body adiposity index (BAI), visceral adiposity index (VAI), lipid accumulation product (LAP), and basal metabolic rate (BMR), among PCOS patients categorized by irregular versus regular menstrual cycles (mc).

The Body Adiposity Index (BAI) showed identical median values for both groups, with 32 for patients with irregular mc. (range: 19-70) and 32 for those with regular mc. (range: 24-45), suggesting that overall body fat relative to height is comparable regardless of menstrual regularity.

In contrast, the Visceral Adiposity Index (VAI) differed significantly. Patients with irregular menstrual cycles had a substantially higher median VAI of 4.2 (range: 1.7-5.8), compared to 2.4 (range: 1.9-3.4) for those with regular cycles. This indicates a greater accumulation of visceral fat in individuals experiencing irregular menstruation.

Similarly, the Lipid Accumulation Product (LAP) was higher in the irregular mc. group, with a median of 119 (range: 30-583), compared to 94 (range: 28-189) in the regular mc. group.

Basal Metabolic Rate (BMR) showed relatively similar median values, with 1422 for the irregular mc. group (range: 1145-1904) and 1470 for the regular mc. group (range: 1217-1768). The ranges, however, indicate considerable individual variability within each category.

The findings from Table 7 highlight that though overall body adiposity (BAI) appears similar, there are notable metabolic differences between PCOS patients based on their menstrual regularity. Patients with irregular menstrual cycles demonstrate higher levels of both Visceral Adiposity Index (VAI) and Lipid Accumulation Product (LAP).

This indicated that irregular menstruation in PCOS is strongly associated with a less healthy metabolic profile, characterized by greater visceral fat accumulation and potentially higher lipid-related risk. The higher VAI and LAP in the irregular cycle group are consistent with the known metabolic disturbances often seen in the more severe phenotypes of PCOS.

The BMR, though slightly different in median, shows substantial overlap in ranges, showing less distinct variation compared to VAI and LAP. These results illustrated the importance of assessing metabolic health, especially visceral adiposity and lipid accumulation, in PCOS patients, particularly those presenting with menstrual irregularities.

Table (3-7). Biochemical evaluation of metabolic incised (Median (mini-max) among PCOS groups based on regularity of mc.

	<i>Irregular mc.</i>	<i>Regular mc.</i>
<i>BAI</i>	32 (19-70)	32 (24-45)
<i>VAI</i>	4.2 (1.7-5.8)	2.4 (1.9-3.4)
<i>LAP</i>	119 (30-583)	94 (28-189)
<i>BMR</i>	1422 (1145-1904)	1470 (1217-1768)

*body adiposity index (BAI), visceral adiposity index (VAI), lipid accumulation product (LAP), and basal metabolic rate (BMR)

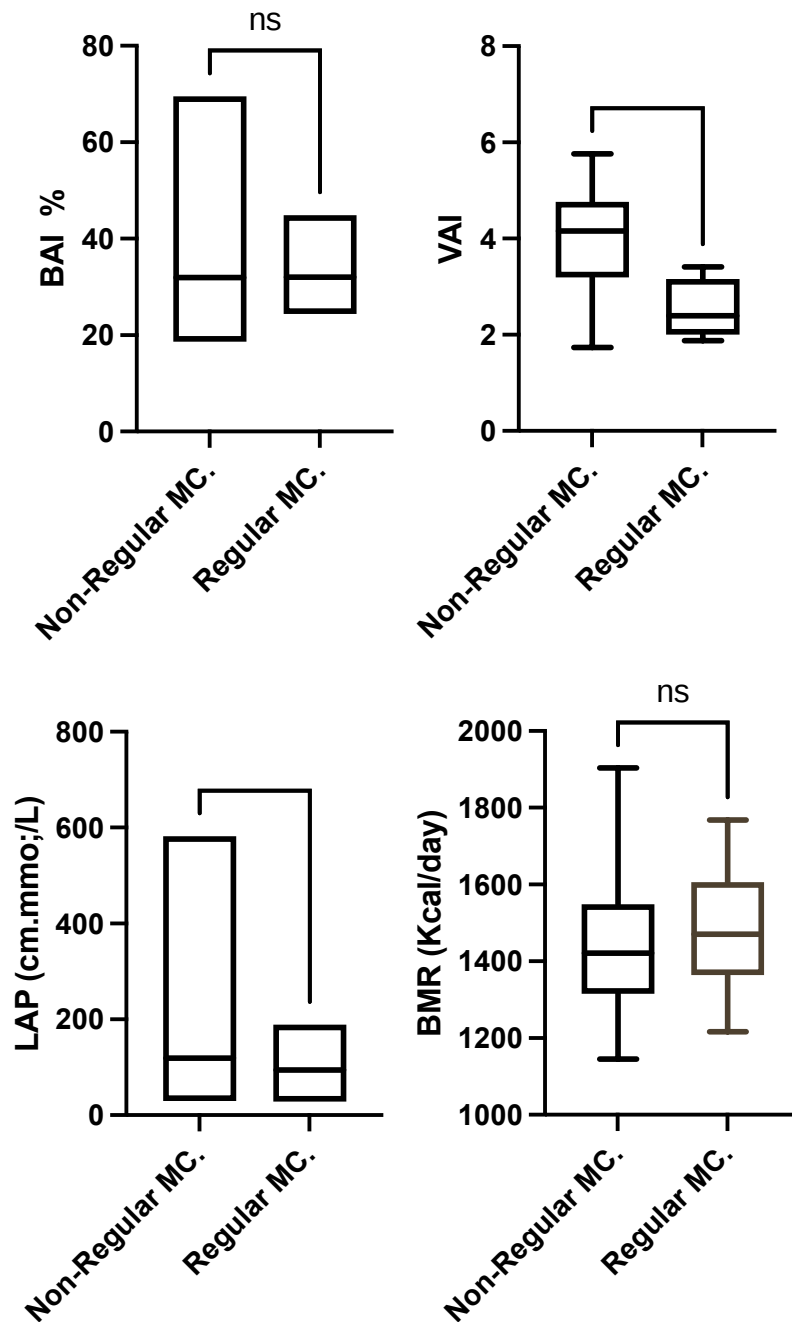


Figure (3-5). The distribution of evaluation metabolic incised among PCOS patients groups based on regularity of mc. (Post Hoc ANOVA test was *: significant at $p \leq 0.05$, **: significant at $p \leq 0.01$, ***: significant at $p \leq 0.001$, ****: significant at $p \leq 0.0001$)

Table (3-8) demonstrated the median (and range) levels of Kallistatin and Zearalenone in PCOS patients, categorized by menstrual cycle (mc) regularity.

Kallistatin levels showed considerable variation across the groups. The Healthy Control group exhibited the highest median Kallistatin level at 117 (range: 16-342). Among PCOS patients, those with regular menstrual cycles had a notably higher median Kallistatin level of 111 (range: 31-121), which is very close to that of healthy controls. In stark contrast, PCOS patients with irregular menstrual cycles displayed a significantly lower median Kallistatin level of 19 (range: 2-271).

For Zearalenone, the median levels were highest in PCOS patients with irregular menstrual cycles, at 6.0 ng/L (range: 2.3-16). Patients with regular menstrual cycles had a lower median of 4.9 ng/L (range: 2.1-10), though the Healthy Control group showed the lowest median Zearalenone level at 4.2 ng/L (range: 2.1-8.2).

PCOS patients with regular menstrual cycles exhibit Kallistatin levels remarkably similar to those of healthy controls, suggesting that favorable Kallistatin levels might be associated with preserved ovarian function and regular ovulation despite a PCOS diagnosis. Conversely, the significantly lower Kallistatin levels observed in PCOS patients with irregular menstrual cycles point to a potential role for Kallistatin as a biomarker for more severe ovulatory dysfunction within PCOS. This could imply that lower Kallistatin contributes to or reflects the underlying physiological mechanisms leading to menstrual irregularity in PCOS.

The elevated median Zearalenone levels in PCOS patients with irregular menstrual cycles compared to both regular mc. PCOS patients and healthy controls is noteworthy. Zearalenone, being a mycoestrogen, can exert estrogenic effects. Its higher presence in irregular cycle patients might reflect an environmental factor contributing to endocrine disruption and exacerbating menstrual irregularities in PCOS.

Table (3-8). Mean level of Kallistatin and Zearalenone (Median (mini-max)) among PCOS patients groups based on regularity of mc.

	<i>Irregular mc.</i>	<i>Regular mc.</i>	<i>Healthy Control</i>
Kallistatin ng/ml	19 (2-271)	111 (31-121)	117 (16-342)
Zearalenone ng/L	6.0 (2.3-16)	4.9 (2.1-10)	4.2 (2.1-8.2)

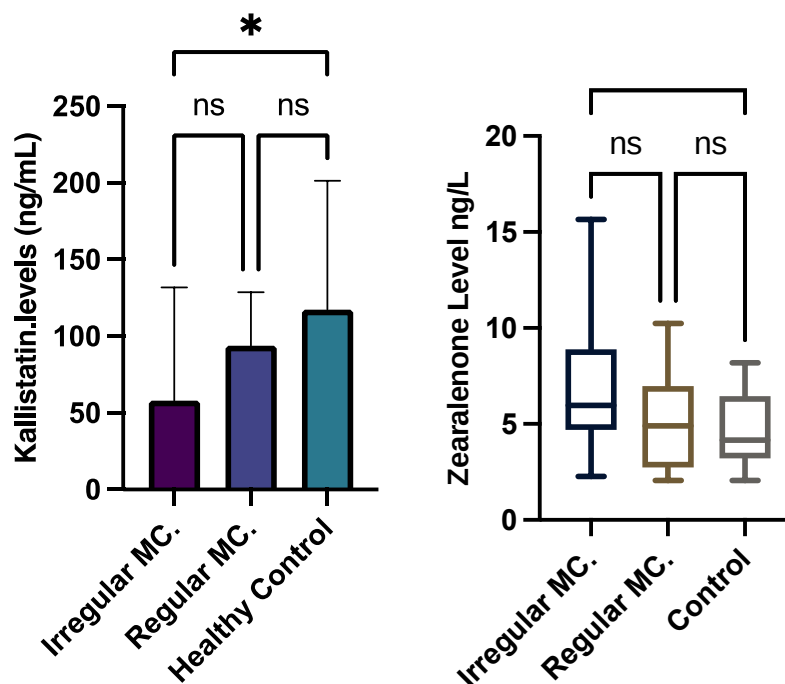


Figure (3-6). The distribution of serum level of Kallistatin and Zearalenone among PCOS patients groups based on regularity of mc. (Post Hoc ANOVA test was *: significant at $p \leq 0.05$, **: significant at $p \leq 0.01$, *: significant at $p \leq 0.001$, ****: significant at $p \leq 0.0001$)**

Table (3-9) presents the median (and range) values of key reproductive hormones, including Luteinizing Hormone (LH), Follicle-Stimulating Hormone (FSH), Prolactin, Testosterone, Sex Hormone Binding Globulin (SHBG), and Estradiol (E2), among four distinct PCOS phenotypes: Classic PCOS (A, HA, PCO), Classic PCOS (A, HA), Ovulatory PCOS (HA, PCO), and Normo-androgenic PCOS (A, PCO).

The Classic PCOS (A, HA, PCO) group exhibited the highest median LH level at 9.8 mIU/mL (range: 1.7-28). The lowest median LH was observed in the Normo-androgenic PCOS (A, PCO) group at 6.0 mIU/mL (range: 1.6-16). Classic PCOS (A, HA) and Ovulatory PCOS (HA, PCO) showed intermediate medians of 6.6 and 7.6 mIU/mL, respectively.

The Normo-androgenic PCOS (A, PCO) group had the highest median FSH at 7.0 mIU/mL (range: 3.8-42). FSH levels were similar across the Classic PCOS (A, HA, PCO) (6.1 mIU/mL), Classic PCOS (A, HA) (6.7 mIU/mL), and Ovulatory PCOS (HA, PCO) (5.8 mIU/mL) groups.

Prolactin levels were highest in the Ovulatory PCOS (HA, PCO) group, with a median of 20 ng/mL (range: 10-32). The lowest median was in the Normo-androgenic PCOS (A, PCO) group at 13 ng/mL (range: 6.6-93).

As expected, groups with hyperandrogenism (HA) showed higher testosterone levels. The Ovulatory PCOS (HA, PCO) group had the highest median Testosterone at 70 ng/dL (range: 9.0-97), followed closely by Classic PCOS (A, HA, PCO) at 64 ng/dL (range: 6.0-140). The Normo-androgenic PCOS (A, PCO) group, by definition, had the lowest median Testosterone at 26 ng/dL (range: 5.0-69).

SHBG levels generally mirrored the inverse of testosterone. The Ovulatory PCOS (HA, PCO) group had the highest median SHBG at 39 nmol/L (range: 26-40), though the Classic PCOS (A, HA, PCO) group had the lowest median SHBG at 18 nmol/L (range: 11-36).

Estradiol levels were relatively high across all phenotype groups, with the Classic PCOS (A, HA, PCO) group showing the highest median E2 at 185 pg/mL (range: 31-185), and the Ovulatory PCOS (HA, PCO) group having the lowest median E2 at 150 pg/mL (range: 33-150).

The results in table (3-9) clearly differentiate the hormonal profiles across the various PCOS phenotypes. These groups generally exhibited higher Testosterone levels and lower SHBG,

consistent with the hyperandrogenism component of their diagnosis. Notably, the Ovulatory PCOS (HA, PCO) group, despite being ovulatory, showed the highest median testosterone and relatively high SHBG, which might indicated a compensatory mechanism or a different androgen metabolism profile compared to the Classic PCOS groups. The Classic PCOS (A, HA, PCO) group displayed the highest LH and lowest SHBG, indicative of more pronounced hormonal dysregulation typically associated with the "classic" form.

As expected, the Normo-androgenic PCOS (A, PCO) group had the lowest Testosterone levels, confirming its definition. Interestingly, this group also showed higher FSH compared to most other phenotypes.

Table (3-9). Reproductive variables (Median (mini-max)) among PCOS phenotype groups

	<i>Classic PCOS (A, HA, PCO)</i>	<i>Classic PCOS (A, HA)</i>	<i>Ovulatory PCOS (HA, PCO)</i>	<i>Normo- androgenic PCOS (A, PCO)</i>
<i>LH (mLU/mL)</i>	9.8 (1.7-28)	6.6 (1.3-19)	7.6 (0.33-20)	6.0 (1.6-16)
<i>FSH (mLU/mL)</i>	6.1 (2.8-14)	6.7 (5.3-9.1)	5.8 (1.3-16)	7.0 (3.8-42)
<i>Prolactin (ng/mL)</i>	18 (7.5-67)	14 (5.2-42)	20 (10-32)	13 (6.6-93)
<i>TESTOSTERON (ng/dL)</i>	64 (6.0-140)	37 (10-143)	70 (9.0-97)	26 (5.0-69)
<i>SHBG (nmol/L)</i>	18 (11-36)	30 (13-32)	39 (26-40)	27 (18-45)
<i>E2 (pg/mL)</i>	185 (31-185)	176 (44-176)	150 (33-150)	173 (31-173)

* A = anovulation (chronic); BMI = body mass index; HA = hyperandrogenism (clinical or biochemical); PCO = polycystic ovaries; PCOS = polycystic ovary syndrome.

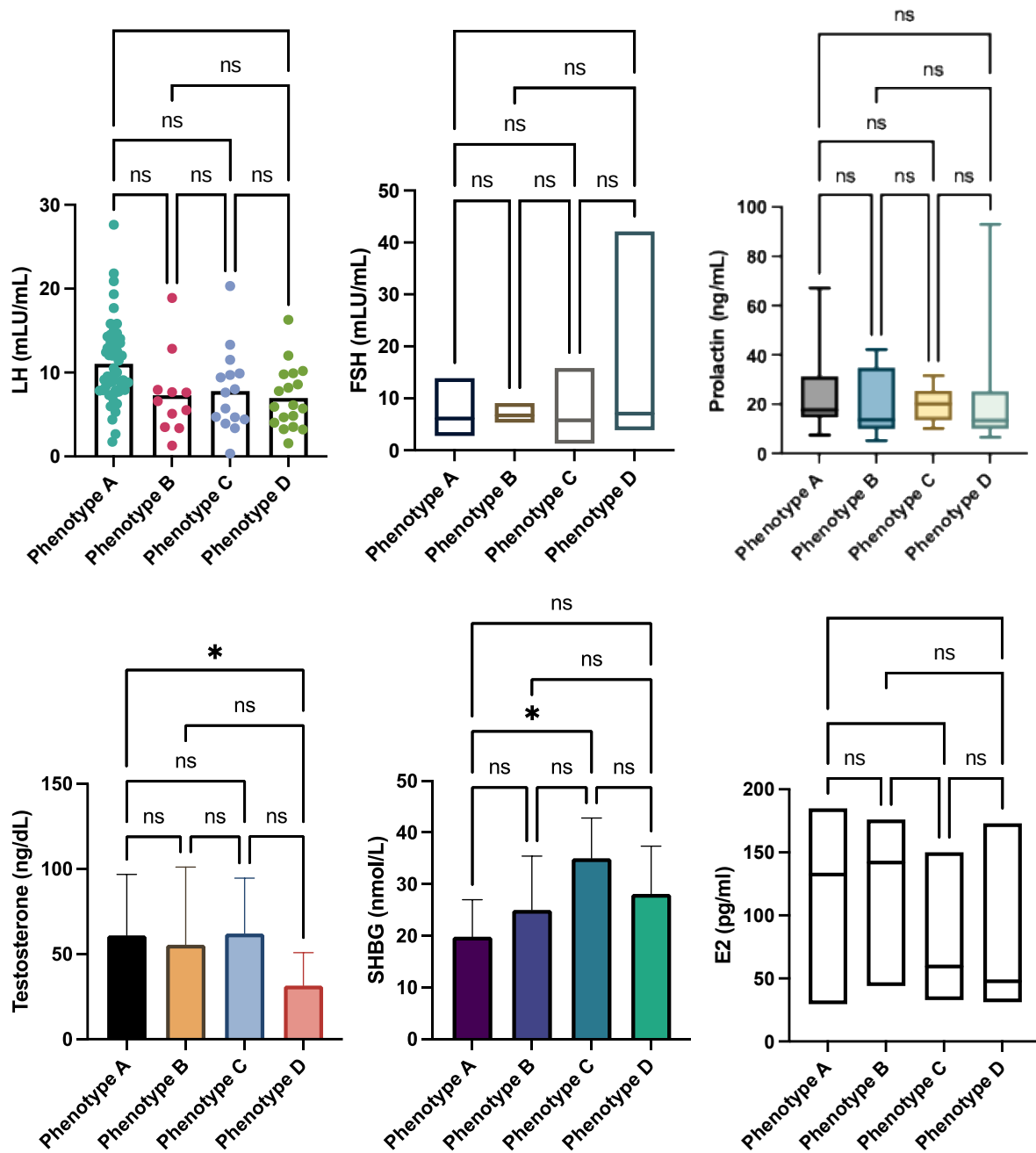


Figure (3-7). The distribution of serum Reproductive variables among PCOS phenotype patients groups (Post Hoc ANOVA test was *: significant at $p \leq 0.05$, **: significant at $p \leq 0.01$, ***: significant at $p \leq 0.001$, ****: significant at $p \leq 0.0001$)

Table (3-10) shown the median (and range) values for metabolic indices, including body adiposity index (BAI), visceral adiposity index (VAI), lipid accumulation product (LAP), and basal metabolic rate (BMR), among four distinct PCOS phenotypes: Classic PCOS (A, HA, PCO), Classic PCOS (A, HA), Ovulatory PCOS (HA, PCO), and Normo-androgenic PCOS (A, PCO).

The median BAI values were relatively consistent across all PCOS phenotypes, ranging from 28 for Classic PCOS (A, HA) to 32 for Classic PCOS (A, HA, PCO) and Normo-androgenic PCOS (A, PCO). This suggests that overall body fat distribution, as measured by BAI, is broadly similar across these different phenotypic presentations of PCOS.

Significant differences were observed in VAI. Both Classic PCOS (A, HA, PCO) and Classic PCOS (A, HA) groups exhibited the highest median VAI at 4.7 (ranges: 2.1-5.8 and 4.1-5.6, respectively). In contrast, the Normo-androgenic PCOS (A, PCO) group had the lowest median VAI at 2.6 (range: 1.7-3.9), followed closely by the Ovulatory PCOS (HA, PCO) group at 2.9 (range: 1.9-5.6). This indicates a higher prevalence of visceral fat accumulation in the classic PCOS phenotypes.

LAP also varied considerably between groups. The Ovulatory PCOS (HA, PCO) group had the highest median LAP at 116 (range: 48-184), followed by Classic PCOS (A, HA, PCO) at 109 (range: 10-583). The Normo-androgenic PCOS (A, PCO) group showed the lowest median LAP at 52 (range: 12-181). The Classic PCOS (A, HA) group had a median LAP of 69 (range: 45-110).

BMR values were relatively similar across the phenotypes. The Classic PCOS (A, HA, PCO) group had the highest median BMR at 1478 (range: 1203-1904), though both Classic PCOS (A, HA) and Normo-androgenic PCOS (A, PCO) groups had the lowest median BMR at 1390 (ranges: 1248-1554 and 1145-1894, respectively).

The Classic PCOS phenotypes (both A, HA, PCO and A, HA) demonstrated significantly higher median VAI compared to the Ovulatory PCOS (HA, PCO) and Normo-androgenic PCOS (A, PCO) groups. This indicated that the presence of both anovulation and hyperandrogenism, with or without polycystic ovaries, is strongly associated with greater visceral fat accumulation

Though Classic PCOS (A, HA, PCO) showed a high LAP, it is notable that the Ovulatory PCOS (HA, PCO) group had the highest median LAP. This indicates that even in ovulatory PCOS, metabolic disturbances related to lipid accumulation can be pronounced. Conversely, the Normo-androgenic PCOS (A, PCO) group consistently presented with the lowest VAI and LAP.

Table (3-10). Biochemical evaluation of metabolic incised (Median (mini-max)) among PCOS phenotype groups

	<i>Classic PCOS (A, HA, PCO)</i>	<i>Classic PCOS (A, HA)</i>	<i>Ovulatory PCOS (HA, PCO)</i>	<i>Normo-androgenic PCOS (A, PCO)</i>
<i>BAI</i>	32 (20-53)	28 (21-70)	31 (24-45)	32 (19-49)
<i>VAI</i>	4.7 (2.1-5.8)	4.7 (4.1-5.6)	2.9 (1.9-5.6)	2.6 (1.7-3.9)
<i>LAP</i>	109 (10-583)	69 (45-110)	116 (48-184)	52 (12-181)
<i>BMR</i>	1478 (1203-1904)	1390 (1248-1554)	1430 (1192-1768)	1390 (1145-1894)

*body adiposity index (BAI), visceral adiposity index (VAI), lipid accumulation product (LAP), and basal metabolic rate (BMR), *A = anovulation (chronic); BMI = body mass index; HA = hyperandrogenism (clinical or biochemical); PCO = polycystic ovaries; PCOS = polycystic ovary syndrome.

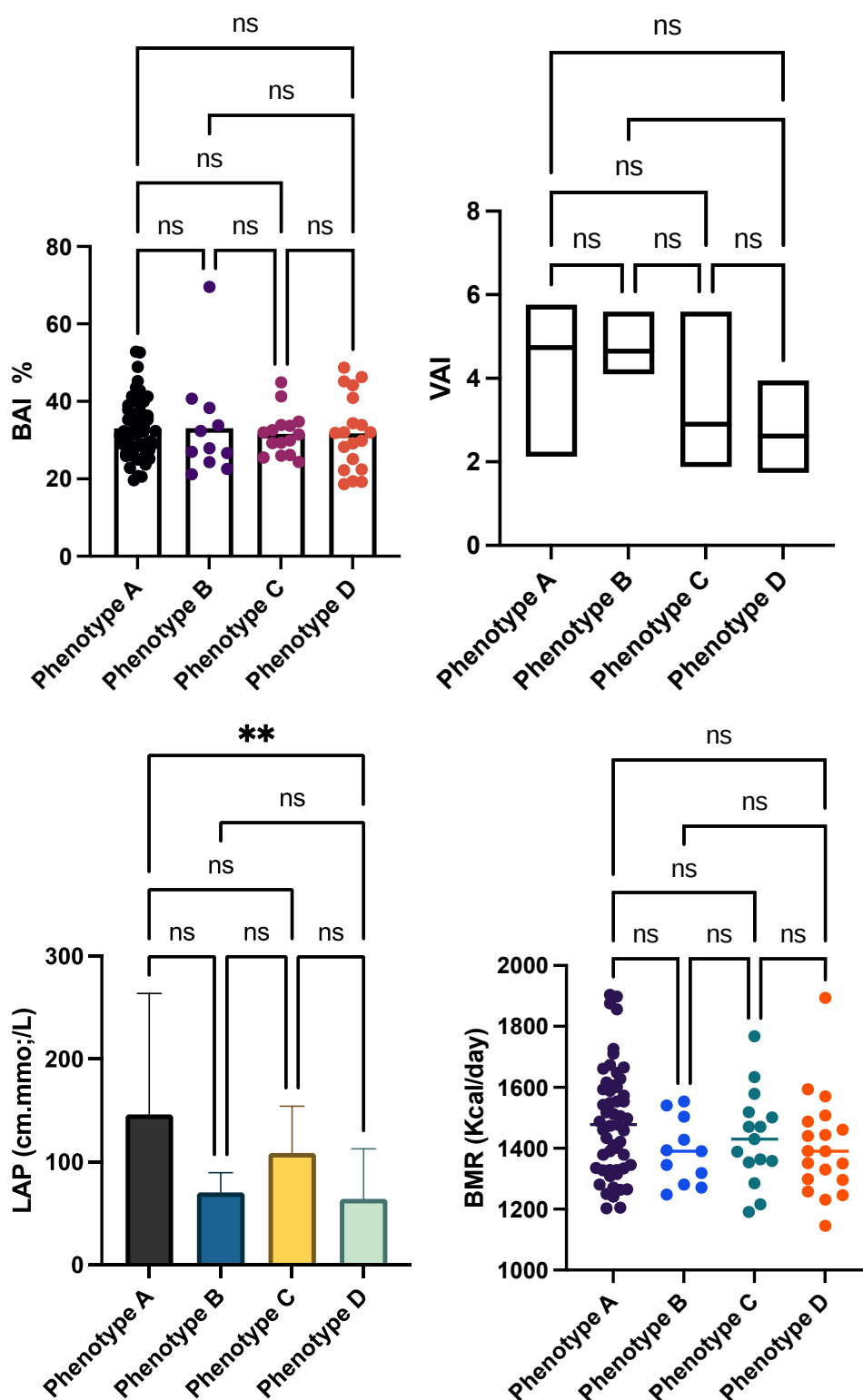


Figure (3-8). The distribution of evaluation metabolic incised among PCOS phenotype patients groups. (Post Hoc ANOVA test was *: significant at $p \leq 0.05$, **: significant at $p \leq 0.01$, ***: significant at $p \leq 0.001$, ****: significant at $p \leq 0.0001$)

Table (3-11) presents the median (and range) levels of Kallistatin and Zearalenone across four distinct PCOS phenotypes: Classic PCOS (A, HA, PCO), Classic PCOS (A, HA), Ovulatory PCOS (HA, PCO), and Normo-androgenic PCOS (A, PCO), alongside a Healthy Control group for comparison.

Kallistatin levels exhibited considerable variation across the groups. The Healthy Control group had the highest median Kallistatin level at 117 (range: 16-342). Among the PCOS phenotypes, the Normo-androgenic PCOS (A, PCO) group displayed the highest median Kallistatin level at 93 (range: 15-131), which was much closer to the healthy control level. The Ovulatory PCOS (HA, PCO) group had a median of 31 (range: 2.6-111). In contrast, both Classic PCOS (A, HA, PCO) and Classic PCOS (A, HA) groups showed significantly lower median Kallistatin levels, at 14 (range: 1.4-71) and 16 (range: 3.4-30), respectively.

For Zearalenone, the Normo-androgenic PCOS (A, PCO) group had the highest median level at 6.5 ng/L (range: 3.3-16). This was followed by the Classic PCOS (A, HA, PCO) group at 4.8 ng/L (range: 2.6-11) and the Classic PCOS (A, HA) group at 4.6 ng/L (range: 3.3-6.7). The Ovulatory PCOS (HA, PCO) group had the lowest median Zearalenone among the PCOS types at 3.6 ng/L (range: 2.1-10), which was comparable to the Healthy Control group's median of 4.2 ng/L (range: 2.1-8.2).

There's a clear trend where Kallistatin levels are lowest in the more severe, classic forms of PCOS (Classic PCOS A, HA, PCO and Classic PCOS A, HA), which are often associated with more pronounced metabolic disturbances and hyperandrogenism. Conversely, Kallistatin levels are significantly higher in the Normo-androgenic PCOS (A, PCO) and Ovulatory PCOS (HA, PCO) groups, with the Normo-androgenic group's levels being particularly close to those of healthy controls. This suggests that higher Kallistatin levels might be protective or indicative of a less severe metabolic and inflammatory profile in PCOS, especially in phenotypes lacking clinical hyperandrogenism or maintaining some degree of ovulatory function.

The distribution of Zearalenone was more varied. Though the Classic PCOS groups show intermediate levels, it's intriguing that the Normo-androgenic PCOS (A, PCO) group, which otherwise appears metabolically healthier (based on Kallistatin), has the highest median Zearalenone level. This might indicate that even in phenotypes with a less overt metabolic or androgenic profile, environmental factors like exposure to mycoestrogens could play a role. The Ovulatory PCOS (HA, PCO) group showing Zearalenone levels similar to healthy controls

could suggest less exposure or different metabolic handling of this compound in this specific phenotype.

Table (3-11). Mean level of Kallistatin and Zearalenone (Median (mini-max)) among PCOS phenotype groups

	<i>Classic PCOS (A, HA, PCO)</i>	<i>Classic PCOS (A, HA)</i>	<i>Ovulatory PCOS (HA, PCO)</i>	<i>Normo-androgenic PCOS (A, PCO)</i>	<i>Healthy control</i>
Kallistatin	14 (1.4-71)	16 (3.4-30)	31 (2.6-111)	93 (15-131)	117 (16-342)
Zearalenone ng/L	4.8 (2.6-11)	4.6 (3.3-6.7)	3.6 (2.1-10)	6.5 (3.3-16)	4.2 (2.1-8.2)

*A = anovulation (chronic); BMI = body mass index; HA = hyperandrogenism (clinical or biochemical); PCO = polycystic ovaries; PCOS = polycystic ovary syndrome.

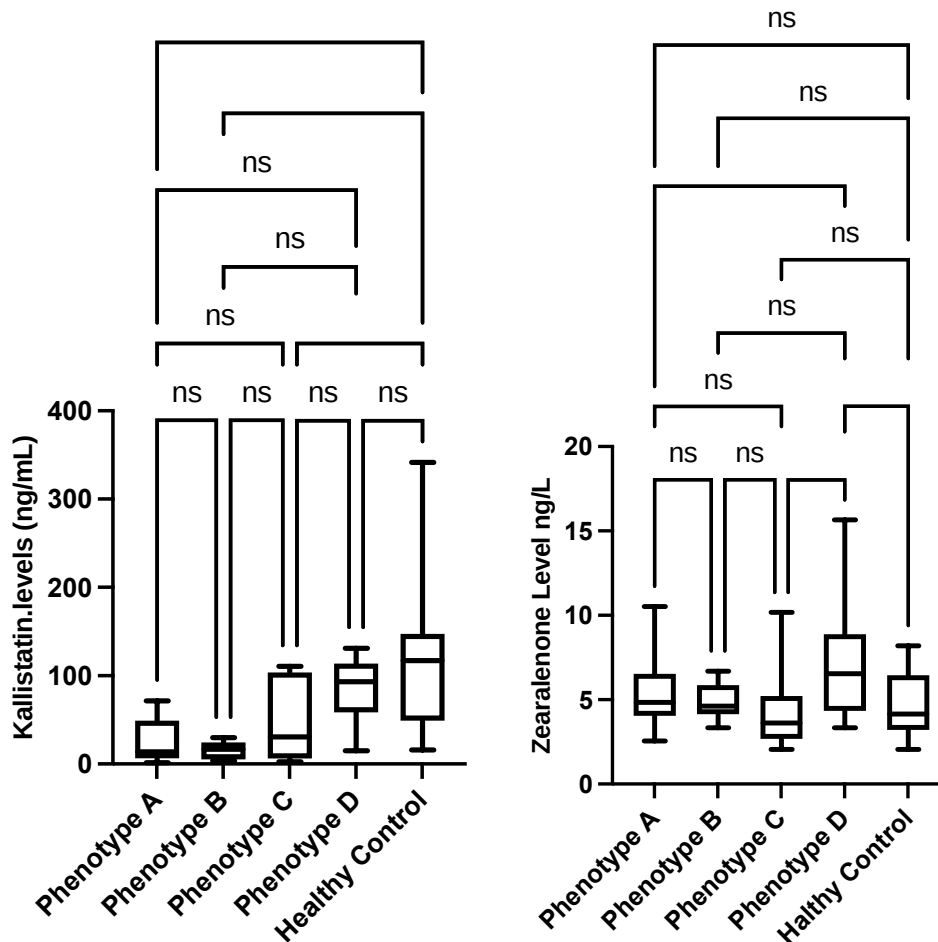


Figure (3-9). The distribution of serum level of Kallistatin and Zearalenone among PCOS phenotype patients groups (Post Hoc ANOVA test was *: significant at $p \leq 0.05$, **: significant at $p \leq 0.01$, *: significant at $p \leq 0.001$, ****: significant at $p \leq 0.0001$)**

Figure (3-10) presents the Receiver Operating Characteristic (ROC) curve for Kallistatin in differentiating PCOS patients of phenotype A (Anovulation, PCO) based on its total and stratification levels. The analysis revealed that Kallistatin demonstrated significant diagnostic utility.

The Area Under the Curve (AUC) for Kallistatin was determined to be 0.88, indicating excellent discriminatory power. At an optimal cut-off value of less than 55.88 ng/mL, Kallistatin achieved a sensitivity of 81% and a specificity of 70% for identifying individuals within the PCOS phenotype A group. The statistical significance of these findings was supported by a P-value of less than 0.001. This suggests that Kallistatin holds promise as a biomarker for the diagnosis or characterization of PCOS patients, particularly within the phenotype A subgroup.

Figure (3-11) presents the Receiver Operating Characteristic (ROC) curve for Kallistatin in distinguishing PCOS patients of phenotype B (Classic PCOS (A, HA)) based on its total and stratification levels. The analysis demonstrated exceptional diagnostic accuracy for Kallistatin in this context.

Kallistatin achieved an impressive Area Under the Curve (AUC) of 0.95, indicating outstanding discriminatory power. At a cut-off value greater than 19.88 ng/mL, Kallistatin provided a high sensitivity of 95% and a strong specificity of 80% for identifying individuals within the PCOS phenotype B group. The statistical significance of these findings was confirmed by a P-value of 0.002. These results strongly suggest that Kallistatin serves as a highly effective biomarker for the diagnosis or characterization of PCOS patients, particularly within the phenotype B subgroup.

Figure (3-12) displays the Receiver Operating Characteristic (ROC) curve for Kallistatin in distinguishing PCOS patients belonging to phenotype C (Ovulatory PCOS (HA, PCO)) based on its total and stratification levels. The analysis indicates that Kallistatin possesses good discriminatory ability for this phenotype.

Kallistatin achieved an Area Under the Curve (AUC) of 0.83, suggesting good diagnostic performance. At a cut-off value greater than 40 ng/mL, Kallistatin yielded a sensitivity of 80% and a specificity of 67% for identifying individuals within the PCOS phenotype C group. The statistical significance of these findings was supported by a P-value of 0.004. These results

suggest that Kallistatin could serve as a useful biomarker for the characterization of PCOS patients, particularly within the Ovulatory PCOS (HA, PCO) subgroup.

Figure (3-13) presents the Receiver Operating Characteristic (ROC) curve for Kallistatin in distinguishing PCOS patients of phenotype D (Normo-androgenic PCOS (A, PCO)) based on its total and stratification levels. The analysis showed limited discriminatory ability for Kallistatin in this particular phenotype.

Kallistatin yielded an Area Under the Curve (AUC) of 0.64, which suggests relatively poor diagnostic performance. At a cut-off value greater than 111 ng/mL, Kallistatin provided a sensitivity of 65% and a specificity of 62% for identifying individuals within the PCOS phenotype D group. The statistical significance of these findings was low, with a P-value of 0.2, indicating that the observed discriminatory power might be due to chance. These results suggest that Kallistatin is not a strong biomarker for characterizing PCOS patients within the Normo-androgenic PCOS (A, PCO) subgroup.

The ROC curve analyses for Kallistatin reveal varying degrees of discriminatory power across the different PCOS phenotypes.

For Phenotype B (Classic PCOS (A, HA)), Kallistatin demonstrated the **highest discriminatory power** in identifying patients in this group, with an outstanding **AUC of 0.95**. It achieved an excellent balance of **95% sensitivity and 80% specificity** at a cut-off of >19.88 ng/mL, along with strong statistical significance (P-value = 0.002). This suggests Kallistatin is a highly effective biomarker for this classic, hyperandrogenic phenotype.

In Phenotype A (Classic PCOS (A, HA, PCO)), Kallistatin also showed **excellent discriminatory power** for this comprehensive classic phenotype, with an **AUC of 0.88**. It offered good diagnostic performance with **81% sensitivity and 70% specificity** at a cut-off of <55.88 ng/mL, supported by high statistical significance (P-value < 0.001).

Regarding Phenotype C (Ovulatory PCOS (HA, PCO)), Kallistatin exhibited **good discriminatory power**, with an **AUC of 0.83**. It achieved **80% sensitivity and 67% specificity** at a cut-off of >40 ng/mL, with statistical significance (P-value = 0.004). Though still useful, its performance was slightly lower than for the classic anovulatory phenotypes.

Though Phenotype D (Normo-androgenic PCOS (A, PCO)), Kallistatin showed **poor discriminatory ability** for the Normo-androgenic PCOS group. Its **AUC was only 0.64**, and it yielded modest performance with **65% sensitivity and 62% specificity** at a cut-off of >111 ng/mL. Crucially, the P-value of 0.2 indicates a lack of statistical significance

Results were shown that Kallistatin appears to be a **highly promising diagnostic or characterizing biomarker for the hyperandrogenic PCOS phenotypes (Phenotype A, B, and C), particularly for Classic PCOS (A, HA) (Phenotype B)**, where it demonstrated exceptional performance. However, its utility is significantly diminished in the **Normo-androgenic PCOS (A, PCO) phenotype (Phenotype D)**, where it failed to show statistically significant discriminatory power. This suggests that Kallistatin's role as a biomarker is strongly tied to the presence of hyperandrogenism and/or the more pronounced anovulatory aspects commonly seen in Classic PCOS presentations.

ROC curve: Phenotype A

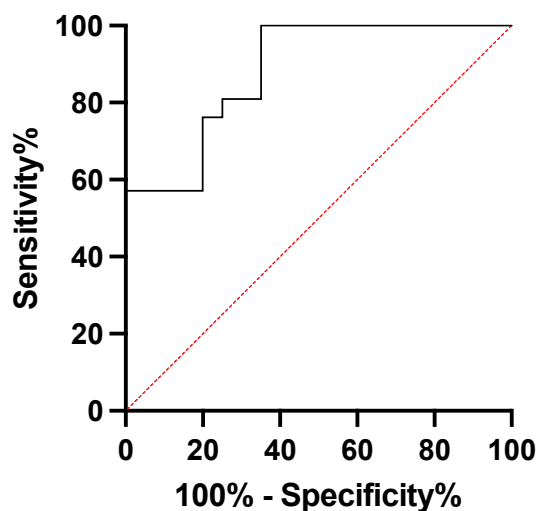


Figure (3-10). ROC curve of Kallistatin PCOS patients/ **phenotype A** group (Total and Stratification levels). (Kallistatin: AUC 0.88, 81% sensitivity, 70% specificity, cut-off <0.55.88 ng/mL, P value was 0.001)

ROC curve: Phenotype B

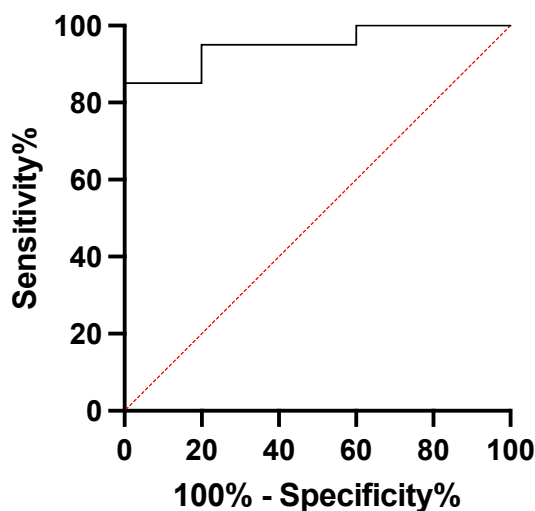


Figure (3-11). ROC curve of Kallistatin PCOS patients/ **phenotype B** group (Total and Stratification levels). (Kallistatin: AUC 0.95, 95% sensitivity, 80% specificity, cut-off > 19.88 ng/mL, P value was 0.002)

ROC curve: Phenotyp C

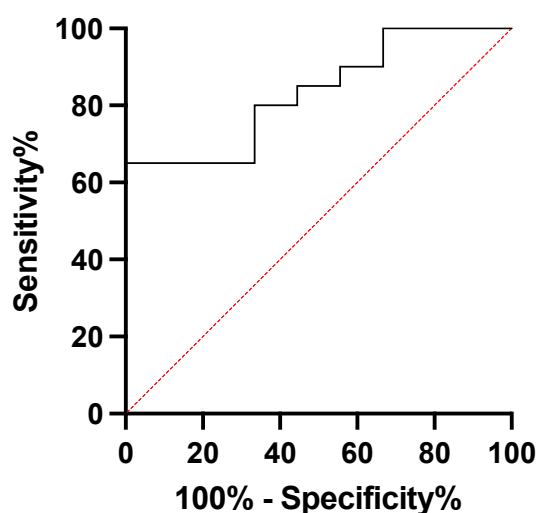


Figure (3-12). ROC curve of Kallistatin PCOS patients/ **phenotype C** group (Total and Stratification levels). (Kallistatin: AUC 0.83, 80% sensitivity, 67% specificity, cut-off > 40 ng/mL, P value was 0.004)

ROC curve: Phenotyp D

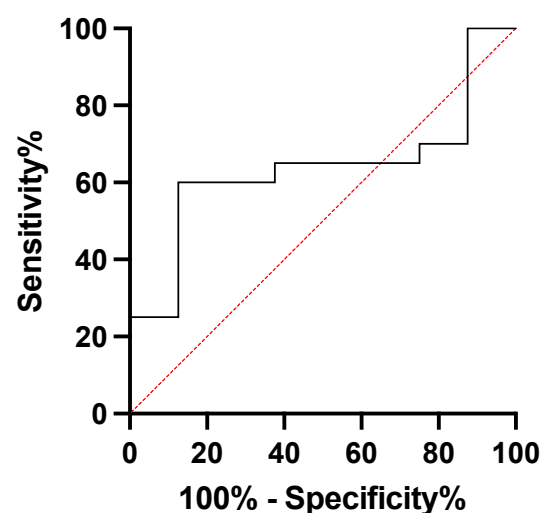


Figure (3-13). ROC curve of Kallistatin PCOS patients/ **phenotype D** group (Total and Stratification levels). (Kallistatin: AUC 0.64, 65% sensitivity, 62% specificity, cut-off > 111 ng/mL, P value was 0.2)

Figure(3-14) illustrates the Receiver Operating Characteristic (ROC) curve for Zearalenone in distinguishing PCOS patients of phenotype A (Classic PCOS (A, HA, PCO)) based on its total and stratification levels. The analysis suggested limited discriminatory ability for Zearalenone in this group.

Zearalenone yielded an Area Under the Curve (AUC) of 0.61, indicating poor diagnostic performance. At a cut-off value greater than 4.305 ng/L, Zearalenone achieved a sensitivity of 72% and a specificity of 60% for identifying individuals within the PCOS phenotype A group. The statistical significance of these findings was low, with a P-value of 0.09, suggesting that the observed discriminatory power might be due to chance. These results indicate that Zearalenone is not a strong biomarker for characterizing PCOS patients within the Classic PCOS (A, HA, PCO) subgroup.

Figure (3-15) displays the Receiver Operating Characteristic (ROC) curve for Zearalenone in distinguishing PCOS patients of phenotype B (Classic PCOS (A, HA)) based on its total and stratification levels. The analysis indicates very limited discriminatory ability for Zearalenone in this group.

Zearalenone yielded an Area Under the Curve (AUC) of 0.60, suggesting poor diagnostic performance, barely better than random chance. At a cut-off value greater than 4.341 ng/L, Zearalenone achieved a sensitivity of 75% and a specificity of 60% for identifying individuals within the PCOS phenotype B group. The statistical significance of these findings was very low, with a P-value of less than 0.4.

Figure (3-16) displays the Receiver Operating Characteristic (ROC) curve for Zearalenone in distinguishing PCOS patients of phenotype C (Ovulatory PCOS (HA, PCO)) based on its total and stratification levels. The analysis suggested extremely limited discriminatory ability for Zearalenone in this group.

Zearalenone yielded an Area Under the Curve (AUC) of 0.60, indicating poor diagnostic performance. At a cut-off value less than 3.67 ng/L, Zearalenone achieved a sensitivity of 53% and a specificity of 61% for identifying individuals within the PCOS phenotype C group. The statistical significance of these findings was very low, with a P-value of less than 0.1

Figure (3-16) displays the Receiver Operating Characteristic (ROC) curve for Zearalenone in distinguishing PCOS patients of phenotype D (Normo-androgenic PCOS (A, PCO) based on its total and stratification levels. The analysis indicated fair discriminatory ability for Zearalenone in this group.

Zearalenone yielded an Area Under the Curve (AUC) of 0.75, suggesting fair diagnostic performance. At a cut-off value greater than 4.163 ng/L, Zearalenone achieved a sensitivity of 77% and a specificity of 50% for identifying individuals within the PCOS phenotype D group. The statistical significance of these findings was acceptable, with a P-value of 0.0033. These results suggest that Zearalenone could be a modest biomarker for characterizing PCOS patients within the Normo-androgenic PCOS (A, PCO) subgroup.

In **Phenotype D (Normo-androgenic PCOS (A, PCO))**, Zearalenone demonstrated its **highest discriminatory power** in this group, with an **AUC of 0.75**. It achieved a reasonable **77% sensitivity** but a lower **50% specificity** at a cut-off of **>4.163 ng/L**. Importantly, the result was statistically significant (P-value = 0.0033). This indicates Zearalenone has a modest, but statistically relevant, ability to characterize this specific phenotype, suggesting a potential role or association in Normo-androgenic PCOS.

Unlike Kallistatin, Zearalenone does not appear to be a strong or consistent biomarker across most PCOS phenotypes. Its discriminatory power is **generally poor for the Classic PCOS phenotypes (A and B) and the Ovulatory PCOS phenotype (C)**, with AUC values close to 0.5 (random chance) and lacking strong statistical significance. Interestingly, Zearalenone shows its **best, albeit still modest, discriminatory ability for the Normo-androgenic PCOS (A, PCO) phenotype (Phenotype D)**, where it reaches an AUC of 0.75 with statistical significance.

ROC curve: ROC of phenotype A

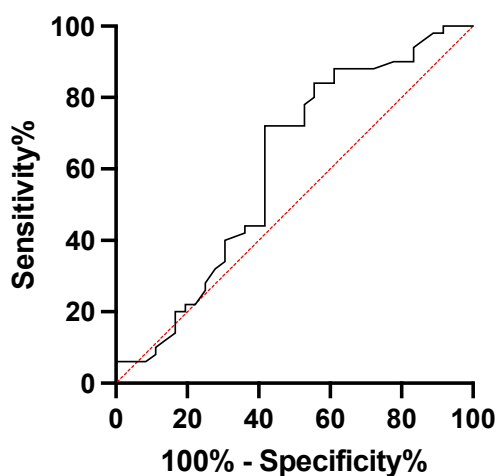


Figure (3-14). ROC curve of Zearalenone PCOS patients/ **phenotype A** group (Total and Stratification levels). (Zearalenone: AUC 0.61, 72% sensitivity, 60% specificity, cut-off > 4.305 ng/L, P value was 0.09)

ROC curve: ROC of phenotype B

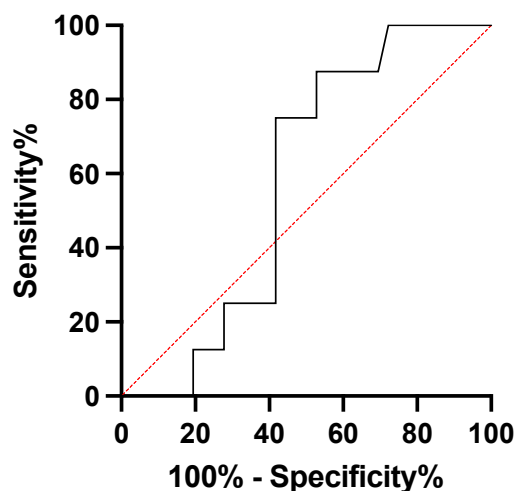


Figure (3-15). ROC curve of Zearalenone PCOS patients/ **phenotype B** group (Total and Stratification levels). (Zearalenone: AUC 0.6, 75% sensitivity, 60% specificity, cut-off > 4.341 ng/L, P value was <0.4)

ROC curve: ROC of Phenotype C

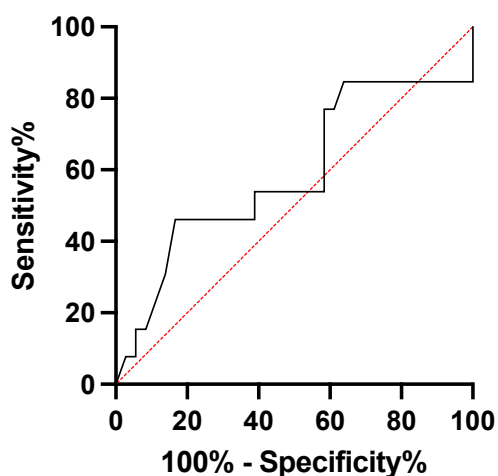


Figure (3-16). ROC curve of Zearalenone PCOS patients/ **phenotype C** group (Total and Stratification levels). (Zearalenone: AUC 0.6, 53% sensitivity, 61% specificity, cut-off < 3.67 ng/L, P value was <0.1)

ROC curve: ROC of Phenotype D

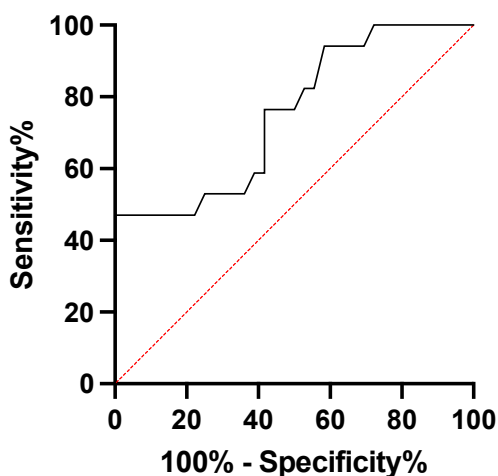


Figure (3-17). ROC curve of Zearalenone PCOS patients/ **phenotype D** group (Total and Stratification levels). (Zearalenone: AUC 0.75, 77% sensitivity, 50% specificity, cut-off > 4.163 ng/L, P value was <0.001)

CHAPTER FOUR

Discussion



Discussion

The reproductive hormone data presented in Table (3-1) showed clear differences between PCOS patients, depending on the type of infertility and marital status. Testosterone levels were significantly higher in women with type 1 infertility (median: 69 ng/dL), though lower values were recorded in the type 2 group (median: 47 ng/dL). This trend is consistent with the known role of hyperandrogenism in PCOS, particularly in patients with chronic anovulation. Chen et al. (Chen, 2020) confirmed that elevated testosterone levels are more common in women with early-onset PCOS and severe infertility.

The opposite trend was observed for sex hormone-binding globulin (SHBG) levels. Patients with type 1 infertility had the highest SHBG concentrations (39 nmol/L), though the lowest mean was observed in the unmarried group (24 nmol/L). Li et al. (Li, 2021) previously described this inverse association between SHBG and testosterone, suggesting that low SHBG levels increase free androgen activity, thereby exacerbating the clinical signs of hyperandrogenism.

Analysis of prolactin (PRO) levels showed moderate elevations in all groups, with a slightly higher mean in the type 2 infertility subgroup (20 ng/ml). Although prolactin is not routinely used in the diagnosis of PCOS, its elevation could reflect subtle disorders of the hypothalamic-pituitary-gonadal axis. Aziz et al. (Azziz, 2020) suggested that mild hyperprolactinemia, often observed in PCOS, may interfere with gonadotropin secretion and ovulatory function, particularly in cases associated with menstrual irregularities. Estradiol (E2) levels also varied, with the group of single women having the highest mean level (64 pg/ml). This could indicate better ovarian function or intermittent ovulation in this group. Rashid et al. (Rashid, 2021) reported similar findings, suggesting that some subtypes of PCOS, particularly in younger women or those with less compromised metabolism, may maintain relatively normal estrogen levels.

Overall, these hormonal patterns highlight the pathophysiological heterogeneity of PCOS. Though type 1 infertility appears to be associated with hyperandrogenism and elevated SHBG levels, type 2 infertility may involve distinct endocrine changes, including prolactin

dysregulation. These findings support the need for phenotypic classification, as recognized by the Rotterdam criteria and reinforced by modern endocrinological frameworks (Teede, 2023). After analyzing reproductive hormone and prolactin levels in different subgroups of PCOS, it was necessary to investigate whether these hormonal changes were associated with metabolic disturbances. Therefore, Table (3-4) focuses on assessing important metabolic parameters, which provides a deeper insight into the systemic nature of PCOS, beyond the reproductive manifestations.

Analysis of the metabolic indices in Table (3-4) revealed significant differences between PCOS patients, depending on the type of infertility and marital status. Though body adiposity index (BAI) values were relatively constant between groups (ranging from 30 to 33), more marked differences were observed in visceral adiposity index (VAI) and lipid accumulation product (LAP). Patients with type II infertility had the highest VAI (3.4) and LAP (94), indicating a greater burden of visceral adiposity and fat accumulation compared to patients with type I infertility and single patients.

These results reflect a better understanding of the metabolic complexity of PCOS. According to Wang et al. (Wang, 2023) visceral fat plays a critical role in inducing insulin resistance and pro-inflammatory states, two factors that exacerbate reproductive and metabolic dysfunction in PCOS. Therefore, a higher VAI in patients with type 2 syndrome could indicate a more severe metabolic profile, despite relatively lower androgen levels (Table 3).

LAP, another lipid-related indicator of metabolic risk, was also more pronounced in the type 2 infertility group. LAP is increasingly recognized as a reliable indicator of cardiometabolic risk in PCOS patients, as demonstrated by (Liu, 2022), who observed a strong association between elevated LAP and increased insulin resistance, particularly in women with oligo-/anovulatory pathologies.

In contrast, basal metabolic rate (BMR) varied slightly between groups, with the type 2 infertility group having the highest mean (1,478 kcal/day). BMR is influenced by multiple factors, including lean body mass and age.

Due to the marked differences in adiposity and metabolic parameters in PCOS patients, the subsequent analysis in Table (3-5) focuses on two novel molecular factors: kallistatin and

zearalenone. Studying the levels of these factors may provide a deeper understanding of the interaction between autoanti-inflammatory proteins and environmental estrogenic toxins in the pathophysiology of PCOS.

The data in Table (3-5) showed significant variation in kallistatin and zearalenone (ZEN) levels across PCOS subgroups. Notably, the control group had the highest mean kallistatin level (66 ng/ml), though the lowest concentration was observed in the unmarried PCOS group (mean: 9.6 ng/ml). In Type 1 infertility patients, the mean kallistatin level was higher (42 ng/ml) than in Type 2 infertility patients (37 ng/ml).

This pattern is consistent with the findings of Zhao et al. (Zhao, 2022), who suggested that decreased kallistatin may be associated with systemic inflammation and endothelial dysfunction in PCOS, especially in women with more advanced metabolic disorders. The significantly lower levels in the unmarried group may indicate a distinct pathological course, perhaps reflecting increased oxidative stress or chronic inflammation not directly related to infertility.

In addition, these results are also consistent with the study by Chen et al. (Chen, 2020), who reported that kallistatin plays a vascular protective and anti-inflammatory role, and that its deficiency may serve as a biomarker of endocrine and metabolic disturbances in PCOS, particularly in phenotypes with marked hormonal imbalance.

For zearalenone, a mycotoxin with known estrogenic activity, the highest mean level was again observed in the unmarried group (7.2 ng/L), followed by the control group (4.8 ng/L), though the control group recorded the lowest level (4.2 ng/L). These findings raise concerns about the potential contribution of environmental endocrine disruptors to the pathophysiology of PCOS, as emphasized by Rashid et al (Rashid, 2021).

ZEN can therefore mimic estrogen and bind to its receptors, potentially disrupting the hypothalamic-pituitary-gonadal axis. Wang et al. (Wang, 2023) highlighted that chronic exposure to ZEN can exacerbate hormonal imbalances and reproductive function, particularly in vulnerable populations. The current findings reinforce this hypothesis, particularly with

higher ZEN levels in the group with the lowest kalistatin levels, suggesting a potential synergistic imbalance affecting endocrine and metabolic stability.

In overview, these findings suggest a dual influence of endogenous (inflammatory/antiprotease imbalance via kalistatin) and exogenous (mycotoxin exposure) mechanisms in the onset of PCOS symptoms. The variability between groups highlights the need for individualized assessment, especially in women with atypical symptoms, such as those who are single or with undiagnosed infertility.

The analysis in Table (3-6) examines the variation of hormonal, metabolic, and biochemical markers in connection to menstrual cycle regularity, based on findings associated with infertile status. This study provides further insights into the hormonal and metabolic variability of PCOS, especially concerning ovulatory function.

Table (3-6) presents a comparative overview of reproductive hormones in PCOS patients, categorized according to menstrual cycle regularity. The data show that patients with irregular cycles tend to have higher mean testosterone levels (61 ng/dL) and lower SHBG levels (29 nmol/L), compared to those with regular cycles (testosterone: 45 ng/dL; SHBG: 43 nmol/L). This hormonal disparity strongly suggests hyperandrogenism and increased androgen bioavailability, characteristics of PCOS in its most severe forms and anovulation.

The observed inverse relationship between testosterone and SHBG is consistent with the findings of Li et al. (Li, 2021), who confirmed that low SHBG promotes high levels of circulating free testosterone, thus exacerbating clinical symptoms such as hirsutism and menstrual irregularity.

Furthermore, FSH and estradiol (E2) levels were significantly higher in women with regular menstrual cycles (FSH: 7.7 mIU/ml, E2: 68 pg/ml) compared to women with irregular menstrual cycles (FSH: 5.8 mIU/ml, E2: 45 pg/ml). This pattern supports the idea that regular menstrual cycles in PCOS patients may indicate persistent or impaired ovulatory activity, as also discussed in Rashid et al. (Rashid, 2021). In this context, increased FSH levels may

enhance follicular growth and estrogen production, potentially stabilizing menstrual cycles despite underlying PCOS.

In contrast, LH levels were elevated in both groups, with the group exhibiting irregular menstrual cycles showing slightly higher levels (8.3 vs. 7.8 mIU/ml), indicating persistent neuroendocrine dysregulation. Prolactin levels exhibited minor variations, yet remained elevated in both groups, indicating a potential subtle role in ovulatory dysfunction among these patients.

Table (3-7) complements the hormonal findings related to menstrual regularity by focusing on metabolic indices. This transition allows for a deeper exploration of how cycle regularity may reflect or interact with underlying metabolic risk in women with PCOS.

As shown in Table (3-7), patients with PCOS and irregular menstrual cycles showed significantly higher metabolic markers, particularly the visceral adiposity index (VAI) and lipid accumulation index (LCI). The mean VAI in the irregular cycle group was 4.2, though the LCI was 119, both significantly higher than the VAI in the regular cycle group (VAI: 2.4; LCI: 94). These findings suggest that menstrual irregularity could be a clinical indicator of a deeper metabolic disorder.

These findings are consistent with those of Liu et al (Liu, 2022), who identified a strong association between increased visceral adiposity, insulin resistance, and ovulatory dysfunction in PCOS. Higher AVF and PAF could reflect increased central obesity, which is known to exacerbate androgen excess and impair folliculogenesis, thus contributing to irregular cycles. Interestingly, body adiposity index (BAI) values were similar between both groups (median: 32), suggesting that total body fat alone may not explain the variation in metabolic risk. Rather, fat distribution, particularly visceral fat, appears to play a larger role, consistent with the findings of (Wang, 2023).

Basal metabolic rate (BMR) showed only minor differences, with slightly higher medians in the regularly cycling group (1470 kcal/day) compared to the irregularly cycling group (1422 kcal/day). Although this finding is not statistically significant, it could indicate differences in

body composition or energy metabolism that influence, or are influenced by, hormonal fluctuations and menstrual cycle regularity.

Taken together, these data suggest that menstrual regularity in women with PCOS is not simply a reproductive indicator but also reflects the underlying metabolic status. Patients with irregular cycles are more likely to have inappropriate adiposity and fat accumulation, reinforcing the multifaceted nature of PCOS.

Building on the observed metabolic disparities, Table (3-8) examines how menstrual regularity correlates with key molecular markers—Kallistatin and Zearalenone—to further elucidate their role in PCOS pathogenesis.

Table (3-8) shows the distribution of kallistatin and zearalenone (ZEN) levels according to menstrual cycle regularity in PCOS patients. Women with irregular menstrual cycles showed significantly lower kallistatin levels (median: 19 ng/ml) and higher ZEN concentrations (median: 6.0 ng/L) compared to women with regular menstrual cycles (kallistatin: 111 ng/ml; ZEN: 4.9 ng/L). The control group consistently showed the best values (kallistatin: 117 ng/ml; ZEN: 4.2 ng/L).

The findings represent the dual influence of inflammatory and environmental factors on reproductive dysfunction in patients with PCOS. Lower kallistatin levels in women with irregular menstrual cycles may indicate increased oxidative stress and subclinical inflammation, both of which are recognized mechanisms that can hinder follicular growth. Zhao et al. (Zhao, 2022) identified kallistatin as an anti-inflammatory molecule that is frequently decreased in women experiencing ovulatory dysfunction and metabolic syndrome.

Thus, elevated ZEN levels in the group of women with irregular menstrual cycles are consistent with the findings of Rashid et al. (Rashid, 2021), who reported that chronic exposure to zearalenone, a mycoestrogen, may disrupt hypothalamic signaling and contribute to menstrual irregularity. This association between low kallistatin and high ZEN may suggest a synergistic

pathway, whereby environmental toxins and decreased protective proteins together exacerbate reproductive and metabolic dysfunction in PCOS.

This section explores the relationship between menstrual regularity and hormonal, metabolic, and molecular changes, focusing on the phenotypes of PCOS. This method facilitates a clearer comprehension of the variations in endocrine, metabolic, and biomarker profiles among different clinical forms of PCOS.

Table (3-9) examines hormonal patterns across the four PCOS phenotypes. The classical PCOS group (A, HA, PCO) showed the highest levels of hormonal dysregulation, including the highest luteinizing hormone (LH) levels (9.8 mIU/ml), the highest testosterone levels (64 ng/dl), and the lowest SHBG levels (18 nmol/l), indicating severe neuroendocrine and androgen dysregulation.

These findings are consistent with the study by Teede et al. (Teede, 2023), which confirmed that phenotypes with both anovulation and hyperandrogenism tended to show more severe hormonal dysregulation. Similarly, the anovulatory PCOS group (HA, PCO) showed the highest testosterone levels (70 ng/dl), but a higher SHBG level (39 nmol/l), suggesting a possible compensatory mechanism or a better regulated androgen availability.

In contrast, the normoandrogenic PCOS phenotype (A, PCO) was characterized by a low testosterone level (26 ng/dl) and a relatively high FSH level (7.0 mIU/ml), possibly indicating a more functional hypothalamic-pituitary-ovarian axis. These differences reflect the variability in PCOS symptoms, with the presence or absence of hyperandrogenism playing a crucial role in determining the hormone profile.

Though Table (3-10) presents a comparison of metabolic indices across phenotypes. The classic PCOS group (A, HA, PCO) and the classic PCOS group (A, HA) showed the highest VAI (4.7) and LAP (Latinospermia), supporting previous findings that metabolic burden is closely associated with a combination of anovulation and hyperandrogenism.

Surprisingly, the anovulatory PCOS group had the highest LAP (116), despite being anovulatory. This suggests that ovulation alone may not protect against metabolic

complications. This observation is consistent with Liu et al. (Liu, 2022), who indicated that metabolic disturbances can persist even in phenotypes without classic reproductive symptoms. On the other hand, the normandrogenic PCOS group showed the lowest VAI (2.6) and LAP (52), indicating a relatively healthier metabolic profile. This supports the idea that this phenotype represents a milder or less metabolically burdensome form of PCOS.

In Table (3-11), the analysis of kallistatin and ZEN levels by phenotype showed a clear trend. The lowest kallistatin level was observed in the classical PCOS group (A, HA, PCO) and the classical PCOS group (A, HA) (16 ng/ml), which are associated with higher inflammatory and androgenic activity. In contrast, the normal androgenic phenotype showed a higher mean kallistatin level (93 ng/ml), almost comparable to the mean of the control group (117 ng/ml), suggesting possible protective mechanisms in phenotypes without hyperandrogenism.

ZEN levels showed a more complex pattern. Unexpectedly, the normal androgenic phenotype registered the highest ZEN concentration (6.5 ng/l), suggesting that environmental estrogen exposure may independently contribute to the development of PCOS, even in the absence of classical endocrine dysfunction. Wang et al. (Wang, 2023) confirmed that these endocrine disruptors can alter gene expression, receptor sensitivity, or inflammatory responses, particularly in phenotypes not traditionally associated with severe androgen excess.

This difference between kallistatin (protective) and ZEN (disruptive) illustrates the multifactorial nature of PCOS and underscores the importance of considering both endogenous inflammatory conditions and external environmental influences when characterizing disease severity across phenotypes.

This study comprehensively examined the reproductive, metabolic, and molecular characteristics of women with polycystic ovary syndrome (PCOS), highlighting the significant variability across phenotypes, infertility types, and menstrual cycle patterns. The results confirmed that PCOS is not a single condition, but rather a spectrum of hormonal and metabolic dysfunctions influenced by both internal and external factors.

Key reproductive hormones, such as testosterone, SHBG, LH, FSH, and estradiol, showed varying expression across PCOS subgroups, with more severe hormonal abnormalities

observed in phenotypes characterized by anovulation and hyperandrogenism. These hormonal trends coincided with changes in metabolic markers, with phenotypes characterized by elevated androgens and menstrual irregularities showing persistently elevated VAI and LAP, suggesting a strong link between reproductive dysfunction and metabolism.

The inclusion of kalistatin and zearalenone provides new insights into the pathophysiology of PCOS. Decreased kalistatin levels were particularly prominent in groups with hyperandrogenism, suggesting its role in systemic inflammation and endothelial dysfunction.

In contrast, elevated ZEN levels—particularly in women with irregular menstrual cycles and a normal androgenic phenotype—suggest the potential influence of environmental estrogens on endocrine imbalance, even in the absence of significant androgen excess.

These findings represent the necessity for phenotypically tailored assessment and management of PCOS. The relationship among hormonal imbalance, metabolic risk, and external factors like ZEN indicates that a comprehensive diagnostic and therapeutic strategy is essential to improve outcomes in this patient group.

CHAPTER FIVE

Conclusions and Recommendations



Conclusions

- This comprehensive study underscores the profound heterogeneity of Polycystic Ovary Syndrome (PCOS) and highlights the differential roles of Kallistatin and Zearalenone as potential biomarkers in its diverse phenotypes and clinical manifestations. Kallistatin consistently emerged as a strong diagnostic and characterization biomarker, particularly for the classic, hyperandrogenic, and anovulatory PCOS phenotypes (A, B, and C), where its significantly lower levels correlated with more pronounced hormonal imbalances and metabolic disturbances, and its ROC curve performance was excellent. This suggests Kallistatin's utility in reflecting both disease severity and underlying metabolic health in these subgroups.
- Conversely, Zearalenone, an environmental mycoestrogen, displayed a more ambiguous role, showing only modest discriminatory power in the Normo-androgenic PCOS (Phenotype D) and poor performance in other phenotypes. Its elevated levels in certain subgroups, like the "Un-married" and irregular menstrual cycle groups, and notably in Normo-androgenic PCOS despite a healthier metabolic profile, suggest its potential as an endocrine disruptor influencing specific aspects of PCOS pathophysiology rather than serving as a general diagnostic marker.
- In essence, the findings advocate for a nuanced approach to PCOS diagnosis and characterization, emphasizing the importance of phenotyping. Kallistatin offers a promising avenue for understanding and potentially targeting the metabolic and inflammatory components of PCOS, particularly in its more severe forms. Though Zearalenone's direct diagnostic utility appears limited, its varied distribution across phenotypes warrants further investigation into its environmental contribution to the heterogeneous manifestations of PCOS.

Recommendations:

This study provides a valuable foundation for understanding the roles of Kallistatin and Zearalenone in PCOS, particularly highlighting the disease's heterogeneity. To further advance this knowledge and translate it into clinical utility, the following future research directions are recommended:

- **Combined Biomarker Panels:** Develop and evaluate diagnostic panels incorporating Kallistatin with other established PCOS biomarkers (e.g., anti-Müllerian hormone, free androgen index, insulin resistance markers) and clinical parameters, potentially leveraging machine learning algorithms, to improve the accuracy of PCOS diagnosis and phenotype classification.
- **Integrated Omics Profiling:** Beyond individual biomarkers, conduct comprehensive multi-omics studies (genomics, transcriptomics, proteomics, metabolomics, epigenomics) on PCOS patients across all phenotypes. This would involve integrating Kallistatin and Zearalenone data with genetic predispositions, gene expression profiles, protein pathways, and metabolic signatures.
- Conduct detailed assessments of dietary intake and environmental exposure to mycotoxins like Zearalenone in PCOS patients versus controls. This could involve food frequency questionnaires and environmental sampling.

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APPENDICES



Appendix-1

The Questionnaire of the study

Name :

Age :

Hight :

Wight :

Waist :

Hip :

Clinical features:

infertility What is the type of infertility?

Is the menstrual cycle regular or not?

Family history:

Medication history:

contraceptive:

When was she diagnosed?

Hormones:

- 1) LH
- 2) FSH
- 3) Prolactin
- 4) Testosterone
- 5) TSH
- 6) SHBG
- 7) estrogen

Lipid profile:

- TG
- LDL
- HDL
- T .cho

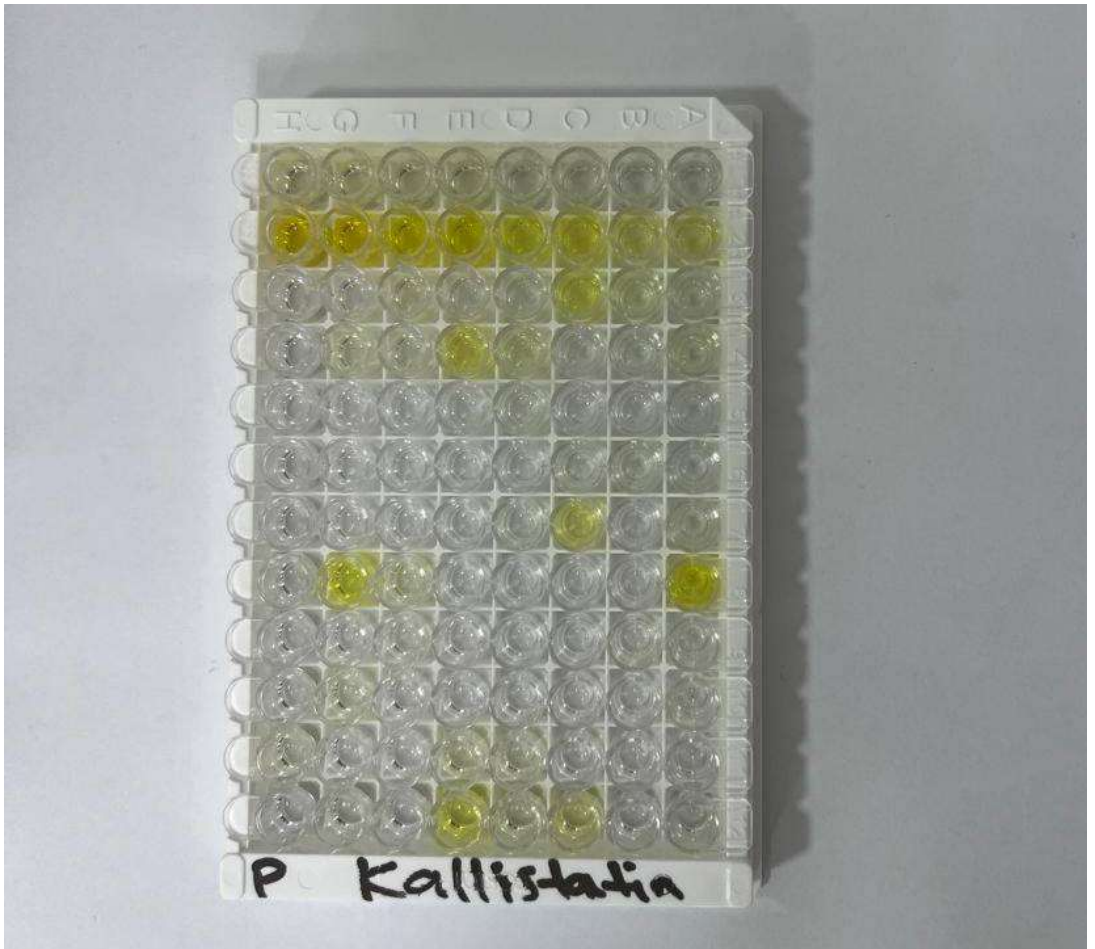
What is the phenotype of polycystic ovary syndrome?

Group A : HA + OD + PCOM

Group B : HA + OD

Group C : HA + PCOM

Group D : OD + PCOM



الخلاصة

(PCOS) متلازمة تكيس المبايض

هي اضطراب هرموني معقد يتميز بخلل في توازن هرمونات الجنس، مما يؤدي إلى اضطرابات في الوظائف التناسلية والتمثيل الغذائي. بينما تم دراسة العوامل الأساسية التي تسبب اضطراب الهرمونات في متلازمة تكيس المبايض بشكل جيد، فإن دور العوامل الناشئة مثل الملوثات البيئية التي تعطل الهرمونات والبروتينات الذاتية الجديدة في تنظيم توازن الزيرالينون، وهو ميكروإستروجين (مادة فطرية ذات نشاط شبيه بالإستروجين)، هرمونات الجنس بدأ يلفت الانتباه معروف بنشاطه الإستروجيني، والكالستاتين، وهو أديبوكاين (بروتين يفرز من الأنسجة الدهنية)، له أدوار في تكوين الأوعية الدموية، التهاب، وتنظيم العمليات الأيضية. لا يزال تأثيرهما المحتمل على البيئة الهرمونية المعقدة في متلازمة تكيس المبايض غير واضح بشكل كامل.

هدفت هذه الدراسة إلى دراسة مستويات الزيرالينون والكالستاتين لدى النساء المصابات بمتلازمة تكيس المبايض، واستكشاف ارتباطهما بمعايير هرمونات الجنس الرئيسية، مما يسلط الضوء على تأثيرهما المحتمل في تنظيم هرمونات أجريت دراسة مقطعية في الفترة من سبتمبر 2024 إلى يوليو 2025 في شملت الدراسة في هذه الفئة من المرضى العراق، وشملت 138 امرأة من مستشفى الولادة وأمراض النساء في كربلاء ومستشفى بغداد التعليمي (مدينة الطب). تم تضمين 98 مريضة تم تشخيصهن بمتلازمة تكيس المبايض و40 امرأة صحيحة صحياً كعينة ضابطة. تم جمع البيانات من خلال استبيان منظم يشمل المعلومات الاجتماعية الديموغرافية، التاريخ الطبي، ونمط الحياة، مع تقييمات سريرية أجريها أطباء متخصصون تم تشخيص 98 امرأة تتراوح أعمارهن بين 18 و44 سنة بمتلازمة تكيس المبايض بناءً على معايير إجماع روتردام لعام 2012، والتي تتطلب وجود اثنين على الأقل من ثلاثة أعراض: فرط الأندروجينية (هرمونات الذكورة) السريرية أو الكيميائية، أو وجود دورات شهرية قليلة أو انقطاع الطمث، و/أو مظهر المبايض المتعدد الكيسات عبر التصوير بالموجات فوق الصوتية. تم تقييم فرط الأندروجينية السريرية بواسطة ظهور الشعر الزائد (تقييم فيريمان-غالوي المعدل)، حب الشباب، أو تساقط الشعر الأندروجيني، بينما شمل فرط الأندروجينية الكيميائية ارتفاع هرمونات الأندروجين في المصل. شملت اضطرابات الدورة الشهرية قلة التبول (دورات تزيد عن 35 يوماً) أو انقطاع الطمث (أقل من 4-6 دورات في السنة). تم تعريف مظهر المبايض المتعدد الكيسات بوجود حجم مبيض أكبر من 10 مل أو وجود 12 ، فرط الأندروجينية (A) كيبساً أو أكثر (2-9 مم). وتم تصنيف المرضى إلى أربعة أنماط سريرية: النمط أ: انعدام الإباضة ، HA ، بدون مظهر المبايض المتعدد الكيسات، النمط ج HA ، A ،: النمط ب (PCO) ، مبايض متعددة الكيسات (HA) ، بدون فرط أندروجينية PCO ، A :مع دورة إباضية، والنمط د PCO

تم جمع بيانات ديموغرافية وسريرية مفصلة، شملت قياسات جسمانية (وزن الجسم، الطول، محيط الخصر/الورك لحساب مؤشر كتلة الجسم، مؤشر كتلة الدهون، مؤشر الدهون الحشوية، مؤشر تراكم الدهون، ومعدل الأيض الأساسي)، أنماط الدورة الشهرية، الحالة الإنجابية، وأعراض متلازمة تكيس المبايض. تم تحليل عينات الدم لفحص ملف الدهون وهرمونات (E2 ، التستوستيرون، الإستروجين LH، FSH) التكاثر.

حققت الدراسة في مستويات الميكويستروجين الزيرالينون والبروتين الأديوكاين الكالستاتين، إلى جانب هرمونات الجنس والمؤشرات الأيضية، لدى 98 امرأة عراقية مصابة بمتلازمة تكيس المبايض عبر أنماط مختلفة (روتريدام أ، ب، ج، د)، وأنواع العقم، وانتظام الدورة الشهرية، مع مقارنتها بـ 40 امرأة صحية. أظهرت النتائج فروقات هرمونية وأيضية مهمة بين مجموعات متلازمة تكيس المبايض أظهر المرضى الذين يعانون من اضطرابات في انتظام الدورة الشهرية والنمط الكلاسيكي فرط الأندروجينية (أ و ب) مستويات أعلى من هرمون ، إلى جانب ارتفاع في مؤشر الدهون (SHBG) التستوستيرون ومستويات أقل من البروتين الرابط لهرمونات الجنس ، مما يشير إلى ملف أيضي وهرموني أكثر حدة (LAP) ومؤشر تراكم الدهون (VAI) الحشوية

كانت مستويات الكالستاتين أقل بشكل مستمر ومهم لدى مرضى متلازمة تكيس المبايض، خصوصاً لدى الذين يعانون من اضطرابات انتظام الدورة والنمط الكلاسيكي الأكثر حدة (أ، ب، ج)، مقارنة بالمجموعة الضابطة الصحية. على العكس، كانت مستويات الكالستاتين أعلى بشكل ملحوظ لدى مرضى متلازمة تكيس المبايض الذين ليس لديهم فرط أندروجينية قدرة تمييز (ROC) (النمط د)، وكانت مشابهة لمستويات المجموعة الضابطة. أظهر تحليل منحنى الاستقبال التشغيلي وقدرة (AUC 0.88 ؛ النمط أ: AUC 0.95) (النمط ب) ممتازة لمستويات الكالستاتين في تشخيص المتلازمة الكلاسيكية (AUC 0.64: د) ، لكن قدرته كانت ضعيفة للنمط النورمو-أندروجيني (AUC 0.83: ج) جيدة للنمط الإباضي

أما مستويات الزيرالينون فكانت بشكل عام مشابهة أو مرتفعة قليلاً في مرضى متلازمة تكيس المبايض مقارنة بالمجموعة الضابطة، مع أعلى المستويات لدى النساء غير المتزوجات ومن يعانون من اضطرابات الدورة الشهرية، ولا سيما في (بين AUC) النمط النورمو-أندروجيني (النمط د). لكن الزيرالينون أظهر قدرة تشخيصية ضعيفة عامة عبر معظم الأنماط (AUC 0.75: د) ، وظهر قدرة تشخيصية متواضعة فقط للنمط النورمو-أندروجيني (0.60-0.61 للأنماط أ، ب، ج).

في الختام، يبدو أن الكالستاتين هو علامة كيميويحيوي قوية محتملة للتشخيص والتوصيف لأنماط متلازمة تكيس المبايض ذات فرط الأندروجينية، مما يعكس الصحة الأيضية والإباضية. أما دور الزيرالينون فهو أكثر تعقيداً، وربما يعمل كمساهم بيئي في بعض مجموعات المتلازمة. تؤكد هذه النتائج على الطبيعة المتغيرة لمتلازمة تكيس المبايض وتبرز أهمية تقييم العلامات كيميويحيوي بناءً على الأنماط لتحسين التشخيص وفهم المرض بشكل أفضل.



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فرع الكيمياء والكيمياء الحياتية



دراسة تأثير الزيرالينون والكاليسنتين في توازن الهرمونات الجنسية لدى مريضات متلازمة تكيس المبايض

الرسالة

قدمت الى مجلس كلية الطب جامعة كربلاء وهي جزء من متطلبات نيل شهادة الماجستير
في الكيمياء السريرية والكيمياء الحياتية

من قبل

شمس الضحى مازن ستار احمد

(بكالوريوس تحليلات مرضية – كلية العلوم الطبية التطبيقية / جامعة كربلاء 2022)

الاشراف

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